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Milk and milk products — Quality control in microbiological laboratories —

Part 1: Analyst performance assessment for colony counts

Lait et produits laitiers — Contrôle de qualité en laboratoires microbiologiques —

Partie 1: Évaluation de la performance des analystes effectuant les comptages de colonies

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ISO copyright office
Case postale 56 • CH-1211 Geneva 20
Tel. + 41 22 749 01 11
Fax + 41 22 749 09 47
E-mail copyright@iso.org
Web www.iso.org

International Dairy Federation
Diamant Building • Boulevard Auguste Reyers 80 • B-1030 Brussels
Tel. + 32 2 733 98 88
Fax + 32 2 733 04 13
E-mail info@fil-idf.org
Web www.fil-idf.org

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 14461-1 | IDF 169-1 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*, and the International Dairy Federation (IDF), in collaboration with AOAC International. It is being published jointly by ISO and IDF and separately by AOAC International.

ISO 14461 | IDF 169 consists of the following parts, under the general title *Milk and milk products — Quality control in microbiological laboratories*:

— *Part 1: Analyst performance assessment for colony counts*

— *Part 2: Determination of the reliability of colony counts of parallel plates and subsequent dilution steps*

Foreword

IDF (the International Dairy Federation) is a worldwide federation of the dairy sector with a National Committee in every member country. Every National Committee has the right to be represented on the IDF Standing Committees carrying out the technical work. IDF collaborates with ISO and AOAC International in the development of standard methods of analysis and sampling for milk and milk products.

Draft International Standards adopted by the Action Teams and Standing Committees are circulated to the National Committees for voting. Publication as an International Standard requires approval by at least 50 % of the National Committees casting a vote.

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All work was carried out by the Joint ISO/IDF/AOAC Action Team, *Statistics of analytical data*, of the Standing Committee on *Quality assurance, statistics of analytical data and sampling*, under the aegis of its project leaders, Dr. H. Glaeser (EU) and Prof. Dr. H. Weiss (DE).

This edition of ISO 14461-1|IDF 169-1, together with ISO 14461-2|IDF 169-2, cancels and replaces IDF 169:1994, which has been technically revised.

ISO 14461|IDF 169 consists of the following parts, under the general title *Milk and milk products — Quality control in microbiological laboratories*:

— *Part 1: Analyst performance assessment for colony counts*

— *Part 2: Determination of the reliability of colony counts of parallel plates and subsequent dilution steps*

Introduction

Every microbiological method consists of several steps that are followed in a specific sequence (sub-sampling, diluting, plating and counting). The final result has a margin of uncertainty that is determined by the variability of all the steps involved.

In order to obtain results with a margin of uncertainty not much larger than what can be expected from the correct application of the method, it is necessary to follow the rules of Good Laboratory Practice (GLP).

The three most important factors in obtaining a correct plate count are

— the homogeneity of the sample material,

— the exactness with which the dilutions are performed, and

— the technique of inoculation and/or counting of the plates.

By homogenizing a sample material very well, making multiple dilution series, and inoculating several plates from the same dilution, it is possible to assess how well a laboratory can perform the colony-count technique, taking into account the expected variability of the method.

A too large variability indicates that at least one of the steps in the performance of the method is out of control. The identification of those steps is done by comparison of the replicate inoculations, the different dilution levels and the dilution series. When the steps with excessive variability have been identified, the necessary measures should be taken to bring these steps under control.

Milk and milk products — Quality control in microbiological laboratories —

Part 1:
Analyst performance assessment for colony counts

1 Scope

This part of ISO 14461|IDF 169 describes a procedure for testing the performance of the colony-count technique within a laboratory by establishing the within-laboratory variability of its technique and identifying those steps that are associated with excessive variability.

The procedure is also suitable for checking the proper observance of Good Laboratory Practice (GLP), which may be a prerequisite for participation in interlaboratory tests of colony-count methods.

EXAMPLE Appropriate test samples are raw milk, pasteurized milk and dried milk.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 648:1977, *Laboratory glassware — One-mark pipettes*

ISO 835-4*, Laboratory glassware — Graduated pipettes — Part 4: Blow-out pipettes*

ISO 4788, *Laboratory glassware — Graduated measuring cylinders*

ISO 7218, *Microbiology of food and animal feedings stuffs — General rules for microbiological examinations*

ISO 8261|IDF 122, *Milk and milk products — Preparation of samples and dilutions for microbiological examination*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

3.1
colony-count technique
counting of the number of microorganisms as determined by the procedure specified in this part of ISO 14461|IDF 169

NOTE The number of microorganisms is expressed per gram or per millilitre of test sample.

4 Principle (see Figure 1)

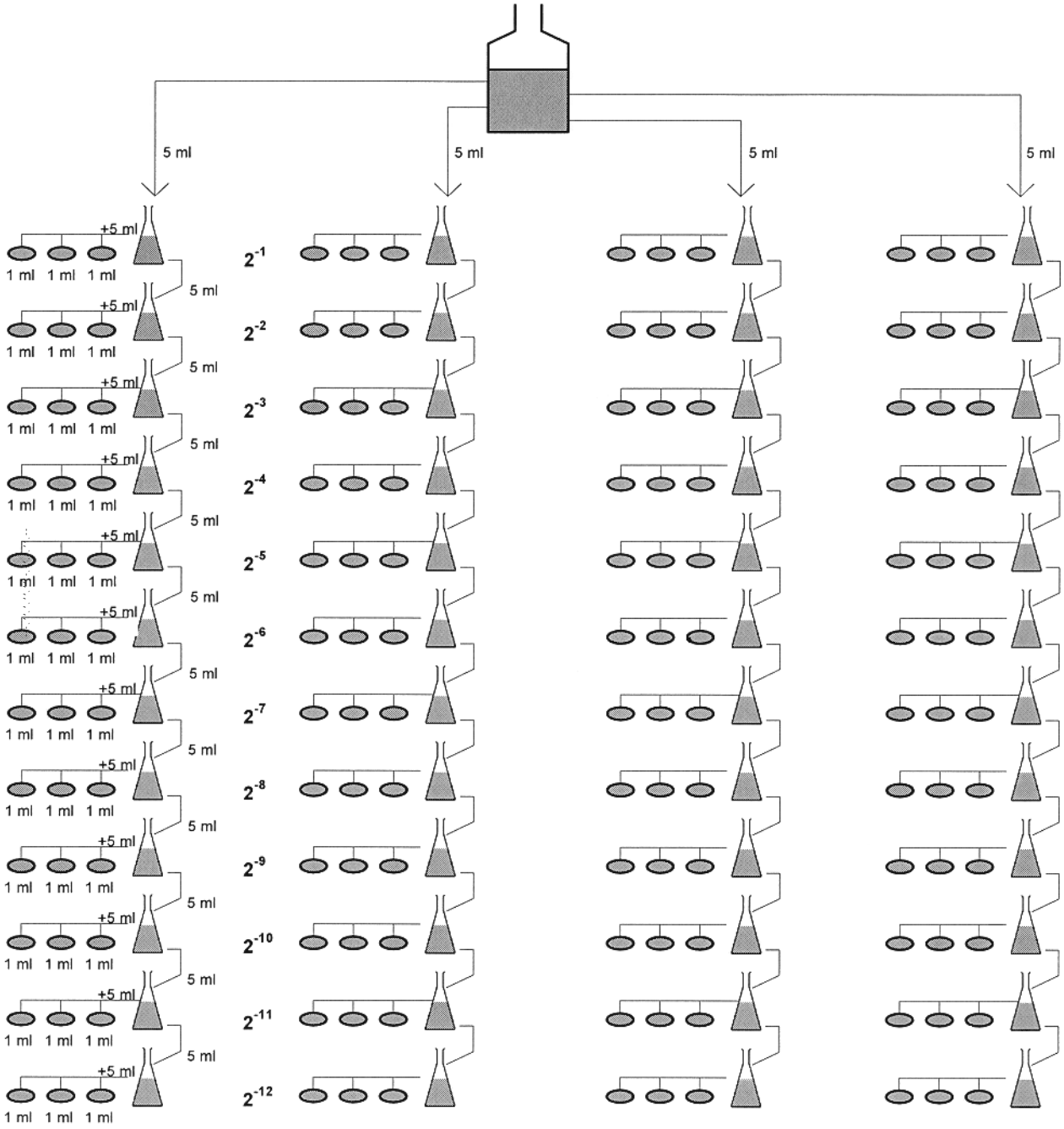


Figure 1 — Quality assurance in the microbiological laboratory: Design of pilot studies for plate count

4.1 A test sample is homogenized then diluted to a suitable working density. (e.g. 500 CFU to 10 000 CFU per millilitre). A suspension is prepared.

4.2 From this first dilution, four dilution series are prepared, each consisting of 12 binary dilution steps.

NOTE Binary (two-fold) dilution steps are used, not decimal (10-fold) dilutions as is the usual practice. With binary dilutions it is possible to count colonies on plates originating from five to six dilutions, and this large number of counts improves considerably the testing of the dilution steps.

4.3 Three parallel plates are poured from each dilution of each series.

4.4 The plates are incubated.

4.5 The sequence of the plates is randomized and the colonies on each plate are counted.

4.6 The counts are tabulated and the “statistical homogeneity” of the counts in two steps is calculated.

4.7 If the values obtained are statistically homogeneous, then the quality of the application of the method is satisfactory and no further evaluation is needed.

4.8 If the results are not statistically homogeneous, an analysis of variance (ANOVA) is performed in order to identify the variation of the results with one or more of the factors that were varied (i.e. dilution series, dilution levels, plating). Further investigations are carried out and the factor(s) identified are adjusted.

NOTE Users will designate the particularly important sources of error in the performance of the method.

5 Diluent, culture media and reagents

The operations described in detail in this clause and in Clause 9 shall either be carried out by one person alone or be divided over a group with clearly defined tasks for each participant.

Use only reagents of recognized analytical grade and distilled water or water of at least equivalent purity, unless otherwise specified. The reagents and the water shall be free from substances that may adversely influence the growth of microorganisms under the test conditions. The culture medium shall be of recognized bacteriological quality. Any dehydrated medium shall be prepared according to the manufacturer's instructions.

5.1 Sodium hydroxide solution or **hydrochloric acid** (approx. 0,1 mol/l), to adjust the pH of the diluent and the culture medium.

5.2 Culture medium: Tryptone-glucose-yeast extract agar, with addition of skimmed milk powder.

5.2.1 Composition

Yeast extract	2,5 g
Tryptic digest of casein (tryptone)	5,0 g
Glucose monohydrate ($C_6H_{12}O_6 \cdot H_2O$)	1,0 g
Skimmed milk powder	1,0 g
Agar	10 g to 15 g [a]
Water	1 000 ml
[a] Depending on the gel strength of the agar.	

In all cases it is necessary to add the skimmed milk powder, even if the dehydrated complete medium is purchased and even if the suppliers consider such an addition unnecessary.

5.2.2 Preparation

For the experiment 2 litres of medium of the same lot will be needed. If a commercial dehydrated complete medium is used, follow the manufacturer's instructions but add the skimmed milk powder. Adjust the pH so that after sterilization it is 7,0 ± 0,2 at about 45 °C.

If the medium is prepared from dehydrated basic components, then dissolve and disperse in preheated water, in the following order: yeast extract, tryptone, glucose and, finally, the skimmed milk powder. Heating the water will assist in the dissolving and dispersion procedure. Add the agar and heat to boiling, while stirring frequently, until the agar is completely dissolved. Alternatively, steam the mixture for about 30 min. Filter the medium through filter paper, if necessary. Adjust the pH so that after sterilization it is 7,0 ± 0,2 at about 45 °C.

Dispense the culture medium in amounts of 250 ml into bottles (6.10). Sterilize all the bottles at one time in the autoclave (6.1) set at 121 °C for 15 min.

Store the prepared medium in the dark at a temperature between 0 °C and 5 °C for no longer than 1 month.

5.3 Diluents: Peptone/salt solution or **quarter-strength Ringer's solution**, from a single lot.

5.3.1 Peptone/salt solution

This is the diluent selected for general use.

5.3.1.1 Composition

Peptone	1,0 g
Sodium chloride (NaCl)	8,5 g
Water up to	1 000 ml

5.3.1.2 Preparation

Dissolve the components in the water, by heating if necessary. Adjust the pH so that after sterilization it is 7,0 ± 0,2 at 25 °C.

5.3.2 Quarter-strength Ringer's solution

5.3.2.1 Composition

Sodium chloride (NaCl)	2,25 g
Potassium chloride (KCl)	0,105 g
Calcium chloride, anhydrous ($CaCl_2$)	0,06 g
Sodium hydrogen carbonate ($NaHCO_3$)	0,05 g
Water up to	1 000 ml

5.3.2.2 Preparation

Dissolve the salts in the water. Adjust the pH so that after sterilization it is 6,9 ± 0,2 at 25 °C.

5.3.3 Preparation of the diluent

Sterilize the diluent by autoclaving, in quantities not greater than 500 ml. Then dispense portions of 90 ml at room temperature into sterile dilution bottles (6.8) using sterile graduated measuring cylinders or other dispensing devices (6.11), and portions of 5 ml into sterile test tubes (6.9) using 5 ml one-mark or graduated pipettes or other dispensing devices (6.12). When emptying a pipette touch the tip against an inclined wall of the container in order to ensure correct delivery.

NOTE Dispensing the portions before autoclaving can lead to unequal evaporation during sterilization, resulting in differences in the final strength of the portions.

Cool and store both the bulk and the dispensed portions of the diluent at a temperature between 0 °C and 5 °C. Use both the bulk and the dispensed portions the next day at the latest.

6 Apparatus and glassware

Sterilize all apparatus that will come into contact with the test sample, the diluent, the dilutions or the culture medium in accordance with ISO 7218 and ISO 8261 | IDF 122.

Usual microbiological laboratory equipment and, in particular, the following.

6.1 Autoclave, capable of operating at 121 °C ± 3 °C.

6.2 Hot-air oven, capable of operating at above 180 °C.

6.3 Incubator, capable of operating at 30 °C ± 1 °C at all points within it.

6.4 pH-meter, with temperature compensation, accurate to ± 0,1 pH units.

6.5 Water baths, capable of operating at 20 °C ± 1 °C, 45 °C ± 1 °C and between 44 °C and 47 °C.

6.6 Lenses, of magnification 2× to 4× and of at least 8×.

6.7 Glass beads, of diameter about 6 mm.

6.8 Dilution bottles, of nominal volume 150 ml to 250 ml, with watertight stoppers, containing 5 to 10 glass beads (6.7). Add the beads before sterilizing the bottles.

6.9 Test tubes, of height about 150 mm and diameter about 15 mm, with stoppers.

6.10 Bottles, of nominal volume 500 ml, with stoppers, for storing 250 ml portions of culture medium.

6.11 Graduated measuring cylinders, with main-point graduations, complying with ISO 4788, or other dispensing devices with a proven equivalent level of accuracy.

6.12 One-mark or **graduated pipettes**, calibrated, capable of delivering 1 ml, 5 ml and 10 ml, complying with ISO 648:1977, class A, or ISO 835-4, or other dispensing devices with a proven equivalent level of accuracy.

6.13 Petri dishes, made of clear uncoloured glass or plastic material, the bottom having an internal diameter of about 90 mm and no irregularities interfering with colony counting.

6.14 Mechanical stirrer, capable of mixing the contents of the test tubes, working on the principle of eccentric rotation (e.g. a vortex mixer).

6.15 Top-loading balance, of sufficient weighing capacity, capable of weighing to the nearest 0,05 g.

7 Sampling

A representative sample should have been sent to the laboratory. It should not have been damaged or changed during transport or storage.

Sampling is not part of the method specified in this part of ISO 14461 | IDF 169. A recommended sampling method is given in ISO 707.

8 Preparation of test sample

8.1 Milk

Agitate the test sample thoroughly by rapidly inverting the sample container 25 times, so that the microorganisms are distributed as evenly as possible. Avoid foaming or allow any foam to disperse. The interval between mixing and removing the test portion shall not exceed 3 min.

8.2 Dried milk

Thoroughly mix the contents of the closed container by repeatedly shaking and inverting it. If the test sample is in the original unopened container and this is too full to permit thorough mixing, transfer it to a larger container, then mix.

9 Procedure

9.1 General

In colony-count methods plates are often partially or completely uncountable due to various reasons (spreading, mould growth, etc.). For the present method, only a limited number of missing values may be tolerated (see 10.1). Too many missing values indicate either that the material used is not suitable for the test, or that the technique is faulty. In such a case, repeat the procedure with another, more suitable sample material or with stricter adherence to the instructions.

9.2 Number of decimal dilution steps

The expected microbiological density of the sample determines the number of decimal dilution steps, as follows:

a) when the expected count is less than 100 000 per millilitre or per gram, make a decimal dilution to 0,1 (one decimal step);

b) when the expected count is between 100 000 and 1 000 000 per millilitre or per gram, make a serial dilution to 10^{-2} (two decimal steps);

c) when the expected count is higher than 1 000 000 per millilitre or per gram, make a serial dilution to 10^{-3} (three decimal steps).

9.3 Preparation of first decimal dilution

9.3.1 Milk

Remove 1 ml of test sample (8.1) with a sterile pipette (6.12) and add to 9 ml of diluent (5.3) (or 10 ml of test sample to 90 ml of diluent, or 11 ml of test sample to 99 ml of diluent). Shake this primary dilution [e.g. 25 times with a movement of about 300 mm for 7 s manually or, using a mechanical stirrer (6.14), for 5 s to 10 s] to obtain a 10^{-1} dilution.

9.3.2 Dried milk

9.3.2.1 Open the container (8.2), remove the amount of test portion required with a spatula and proceed as indicated in 9.3.2.2. Immediately close the container again.

9.3.2.2 Weigh 10 g of test sample into a suitable glass vessel (e.g. a beaker) and then add the powder to the dilution bottle containing a suitable diluent (5.3). Alternatively, weigh 10 g of test sample directly into the bottle with the diluent. To dissolve the test sample, swirl slowly to wet the powder then shake the bottle (e.g. 25 times with a movement of about 300 mm, for about 7 s). A peristaltic-type blender may be used as an alternative to shaking. Allow to stand for 5 min, shaking occasionally.

9.4 Preparation of further decimal dilutions

Prepare further dilutions in accordance with ISO 8261 | IDF 122.

9.5 Melting the medium

Before starting the operations described in 9.6, melt the culture medium (5.2) and cool it in the water bath (6.5) set at between 44 °C and 47°C. Check the temperature of the medium by placing a thermometer into a 250 ml portion of agar (e.g. water agar) in a separate container, which is identical to that used for the medium. Pour the molten agar within 2 h after melting.

9.6 Preparation of binary dilutions and inoculation of the medium

9.6.1 First dilution series (S_1)

Take 12 dilution test tubes (6.9) with 5 ml of diluent from cold storage (5.3.3).

Make serial binary dilutions (D_1, D_2, …) by transferring with a fresh pipette 5 ml of the suspension from the previous dilution (9.4) into a tube with 5 ml of diluent. Mix the suspension 5 times during 5 s with the stirrer (6.14) before each transfer. The first inoculum is taken from the last decimal dilution bottle (9.4), which is immediately placed back in the refrigerator.

Before starting the next binary dilution series, inoculate three Petri dishes (P_1, P_2 and P_3) from each of the twelve dilutions using 1 ml one-mark or graduated pipettes (6.12). Use a fresh sterile pipette for each dilution level.

After inoculating all plates of the series (S_1), pour 12 ml to 15 ml of molten and tempered (44 °C to 47°C) culture medium (9.5) into each Petri dish in the same working order as the inoculation. Mix the medium carefully with the inoculum by rotating the Petri dishes sufficiently to obtain evenly dispersed colonies after incubation. Allow the mixture to solidify by leaving the Petri dishes to stand on a cool horizontal surface.

9.6.2 Subsequent dilutions (S_2, S_3 and S_4)

After completion of the first series of dilutions and plating, prepare the second, third and fourth dilution series (S_2, S_3 and S_4) similarly, starting each time with the mixing of the contents of the last decimal dilution bottle (9.4) stored in the refrigerator in the meantime. Use two or three 250 ml portions of molten culture medium for plating each dilution series and discard the rest.

9.7 Incubation

Invert the prepared dishes and place them in the incubator (6.3) set at 30 °C for 72 h ± 2 h. Do not stack the dishes more than three high. Mark the position of each dish in a stack (low – middle – high).

NOTE This information may be useful if it turns out that the variability between the plates is too large and a possible stacking effect is to be investigated.

Separate stacks of dishes from one another and from the walls and top of the incubator. Do not leave trays in the incubator.

9.8 Randomization and counting of colonies

9.8.1 Randomization

Do not count the plates in order of the dilutions or grouped according to the dilution step, as this may result in an underestimation of the variability because the person who performs the counting will have an idea of what to expect. Therefore, the plates shall first be examined and randomized as described in this subclause by a person not involved in the counting in 9.8.2.

Starting from the most diluted series, select “countable” dilutions, i.e. dilutions for which the expected average count is between 5 and 300 colonies per plate.

Recode all plates of the countable dilutions, using random numbers in order to randomize the countable plates over all series and dilutions. See Table 1 for one such set of numbers; other random sequences from 1 to 144 may also be used. Remove the original markings of the plates; the use of removable adhesive labels is recommended. Designate the uncountable plates with a minus sign in the protocol (see Table 2).

Table 1 —Example of random recoding of the plates for use with Table 2

Dilution series					
Dilution steps	**Plate**	S_1	S_2	S_3	S_4
2^{-1}	1	129	2	82	96
	2	29	21	105	46
	3	143	80	6	35
2^{-2}	1	140	130	124	122
	2	100	65	135	45
	3	93	88	107	87
2^{-3}	1	127	117	115	106
	2	108	138	18	20
	3	89	10	63	75
2^{-4}	1	50	123	49	61
	2	17	97	72	99
	3	32	104	26	128
2^{-5}	1	52	13	64	5
	2	22	14	40	34
	3	134	25	37	70
2^{-6}	1	69	113	11	1
	2	136	109	48	78
	3	95	31	19	28
2^{-7}	1	4	74	59	57
	2	79	67	39	71
	3	73	51	141	23
2^{-8}	1	92	12	55	62
	2	30	66	133	81
	3	27	131	91	121
2^{-9}	1	101	144	15	58
	2	36	98	116	24
	3	16	83	56	47
2^{-10}	1	68	94	120	142
	2	43	38	119	132
	3	90	41	85	33
2^{-11}	1	125	139	137	111
	2	114	126	53	8
	3	54	9	76	86
2^{-12}	1	102	42	3	103
	2	77	44	110	7
	3	84	112	118	60

9.8.2 Counting of colonies

Examine the plates under subdued light. To facilitate counting, a suitable lens (6.6) and/or a tally counter may be used. Take care to avoid mistaking particles of undissolved sample or precipitated matter in dishes for pinpoint colonies. Examine doubtful objects carefully, using a lens of higher magnification (6.6) where required, to distinguish colonies from foreign matter.

Enter the colony counts as given in Table 2.

Deviations from normal appearance that might influence the evaluation, such as overgrown or partially overgrown plates, should be indicated in the table (Figure 2).

Coding and counting shall take place on the day the incubation is completed.

Table 2 — Example of a note sheet for colony counts, for use during the counting of the plates recoded according to Table 1 in numerical order

Plate	Count	Plate	Count	Plate	Count	Plate	Count
1		37		73		109	
2		38		74		110	
3		39		75		111	
4		40		76		112	
5		41		77		113	
6		42		78		114	
7		43		79		115	
8		44		80		116	
9		45		81		117	
10		46		82		118	
11		47		83		119	
12		48		84		120	
13		49		85		121	
14		50		86		122	
15		51		87		123	
16		52		88		124	
17		53		89		125	
18		54		90		126	
19		55		91		127	
20		56		92		128	
21		57		93		129	
22		58		94		130	
23		59		95		131	
24		60		96		132	
25		61		97		133	
26		62		98		134	
27		63		99		135	
28		64		100		136	
29		65		101		137	
30		66		102		138	
31		67		103		139	
32		68		104		140	
33		69		105		141	
34		70		106		142	
35		71		107		143	
36		72		108		144	

NOTE Plates not counted or uncountable are noted as "—", plates without colonies are noted as "O".

10 Statistical evaluation

10.1 Adequacy of the data set

NOTE In the tables and formulae, the symbols i, j and k are indices denoting the dilution series S, dilutions D and plates P, respectively.

When all three parallel plates of any suspension are uncountable, then discard the counts for that dilution in the other three dilution series.

The experiment can be evaluated statistically if at least five successive binary dilution levels are available. Altogether about 5 % of missing counts [i.e. 3 plates out of 60 (5 dilution levels), or 4 plates out of 72 (6 dilution levels)] can be tolerated, except when they include a whole set of three parallel plates. In this case all the plates of the corresponding dilution shall be discarded.

Additionally, the expected average counts of the five or six acceptable dilution levels shall lie within the range from 5 to 300 colonies per plate.

If these conditions are not met, then the data set is considered incomplete. Repeat the procedure, choosing a more suitable sample material (if there are too many uncountable plates) or following more closely the instructions given, or both.

10.2 Evaluation of the complete data set (see Figure 2)

10.2.1 Tabulation of the counts

Each count belongs to a specific dilution series S_i (S_1, S_2, S_3 or S_4), to a specific dilution D_j (D_1, D_2,….D_6, where D_1 is the lowest dilution for which countable plates were obtained), and to a specific plate P_k (P_1, P_2 or P_3). For a better overview of the results, reorganize the counts of Table 2 according to dilution series S_i, to dilution D_j and to plate P_k as shown in Table 3.

Table 3 — Tabulation of the counts

S_i	D_j	P_k		
		P_1	P_2	P_3
S_1	D_1	C_{111}	C_{112}	C_{113}
	D_2	C_{121}	C_{122}	C_{123}
	D_j	C_{1j1}	...	...
	...	...	...	...
S_2	D_1	C_{211}	...	...
	D_2	...	...	...
	D_j	...	...	...
	...	...	...	...
S_i	D_j	...	C_{ij2}	...
...	...	...	...	...
NOTE The entry C_{123} is the count from the third plate of the first series for the second dilution.				

10.2.2 Testing the homogeneity of the plating: G_P^2-test

The first step in the statistical evaluation of the counts (see Figure 2) consists of the determination of the magnitude of the statistical homogeneity of the replicate plates. This test is performed to detect results that are "too good"; i.e. to detect the case in which the variability of the counts between parallel plates P_1, P_2 and P_3 is smaller than expected ("under-dispersion"). This can, for example, be caused by insufficient randomization of the plates before counting.

This statistical homogeneity is a single number that is obtained as follows (see Annex A for more information on the G_P^2 test and also the example in Table 6).

a) Determine for each group of three parallel plates the mean value $C_{ij\cdot}$ (e.g. C_{12} = (C_{121} + C_{122} + C_{123})/3, the mean of the results of one row in the table).

b) Calculate for each plate with count C_{ijk}, the value $C_{ijk} \cdot \ln (C_{ijk}/C_{ij})$, with C_{ij} the mean for that set of parallel plates.

c) Sum the three values in each row (the parallel plates) and multiply by two to obtain the individual G^2 values for each set of parallels. Write these in the table. Sum those values over all the dilutions and series to obtain the test value:

$$G_P{}^2 = 2\left[\sum_i^s \sum_j^d \sum_k^p \left(C_{ijk} \ln \frac{C_{ijk}}{C_{ij}} \right)\right]$$

where s is the number of the dilution series, d is the number of dilution steps, and p is the number of plates.

NOTE An alternative form is given in Annex A, Equations (A.6) and (A.7).

d) Compare the value of G_P^2 with the tabulated values of χ^2 (chi-squared) in Table 4 for 40 degrees of freedom (df = 40) in the case of 5 dilutions, and 48 degrees of freedom (df = 48) in the case of 6 dilutions. If some results are missing (see 10.1), subtract the number of missing results from the df before comparing with the values in Table 4.

If the value of G_P^2 is *less* than the tabulated 0,995 probability value, then the counts of the parallel plates show less variation then could be expected: the values are statistically too homogeneous. The analyst shall confirm this by randomly recoding the plates and recounting them. In the case of confirmation, the experiment shall be repeated with stricter adherence to the procedure.

If the value of G_P^2 is *greater* than the tabulated probability value (P) of 0,995 %, then the variability between the parallel plates is not too low. Proceed with the next test in 10.2.3.

If the value of G_P^2 is *considerably greater* than the tabulated 0,01 probability value, the parallel plates show larger variation than is to be expected. This observation should be kept in mind for possible future use in the interpretation of great overall variation in later stages of the evaluation.

Table 4 — Values for χ^2 (P = 0,01 and P = 0,995)

df	$\chi^2_{0,01}$	$\chi^2_{0,995}$	df	$\chi^2_{0,01}$	$\chi^2_{0,995}$
37	59,89	18,59	44	68,71	23,58
38	61,16	19,29	45	69,96	24,31
39	62,43	20,00	46	71,20	25,05
40	63,69	20,71	47	72,44	25,78
			48	73,68	26,51

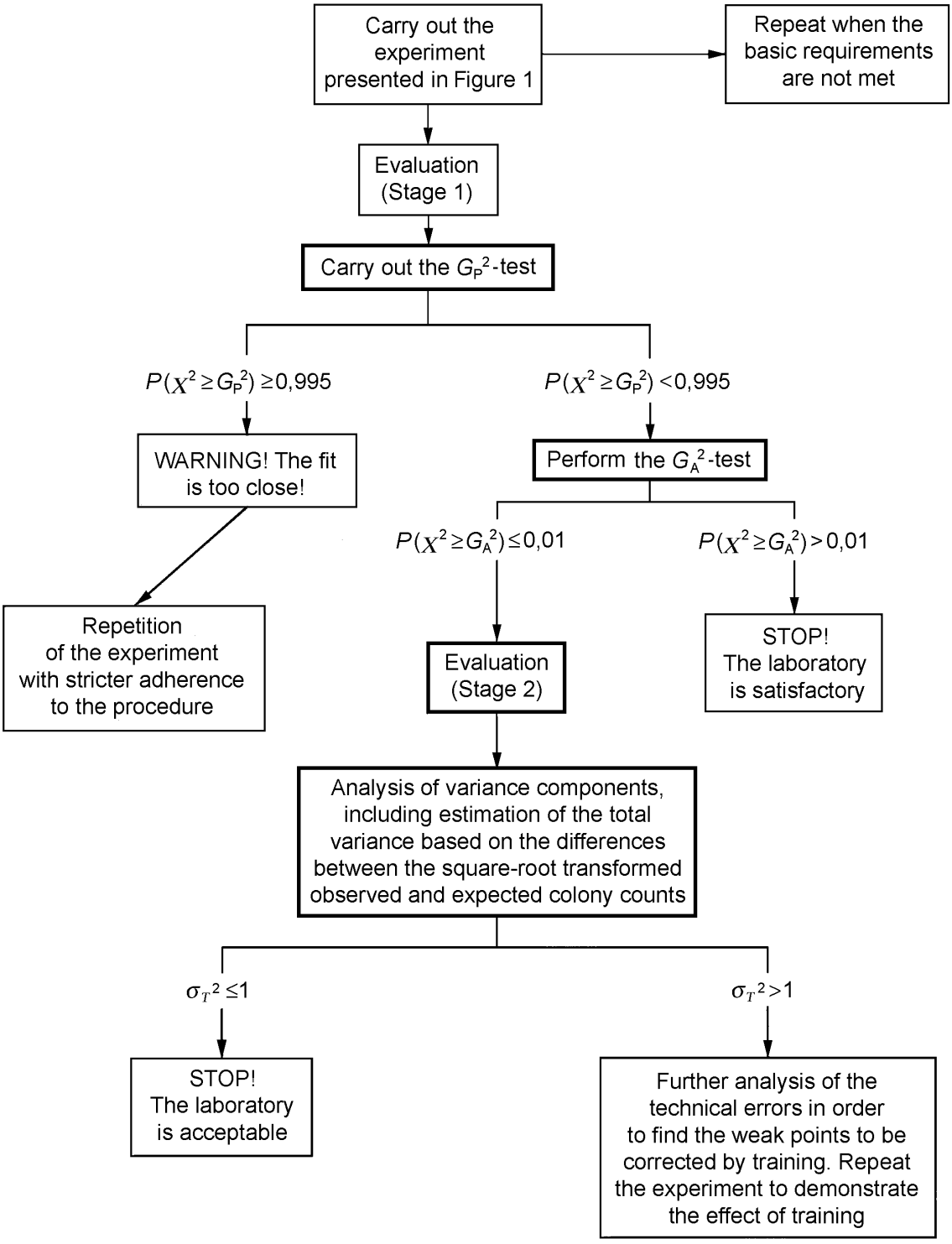


Figure 2 — Flow diagram of the statistical analysis of a laboratory performance study of the plate count

10.2.3 Testing the overall homogeneity of the counts

10.2.3.1 The G_A^2-test

The second step (see Figure 2) consists of the determination of the magnitude of the statistical homogeneity of the whole data set. In this test, the counts are compared with their expected values, taking into account the effects of the dilutions.

This overall statistical homogeneity is a single number that is obtained as follows (see also Example).

a) Determine the total number of colonies in the table (ΣC_{ijk}) and in the total sample volume (ΣV_{ijk}) for which colonies were counted.

b) The sample volume of the highest dilution step D_d for which colonies were counted (D_5 or D_6) is taken as the unit volume (V_d = 1). For each lower dilution step D_i the corresponding sample volume V_i can be determined as

$$V_i = V_d \cdot 2^a$$

where a is the number of binary dilutions between D_i and the highest dilution D_d.

EXAMPLE Suppose the highest dilution for which valid counts were obtained is D_5, corresponding to a dilution of 2^{-10}; the sample volume of this dilution is taken as the unit volume (V_5 = 1). The corresponding volume V_3 for dilution D_3 (2^{-8}), two binary dilution steps from D_5, is calculated as $V_3 = V_5 \times 2^2 = 4$ unit volumes V_5.

1) Calculate the expected number of colonies per unit of sample volume $e = \Sigma C_{ijk} / \Sigma V_{ijk}$ and from this calculate the expected number of colonies $E(C_{ijk}) = e \cdot V_{ijk}$ for each dilution.

2) Calculate for each plate with count C_{ijk}, the value $C_{ijk} \cdot \ln [C_{ijk}/E(C_{ijk})]$.

3) If $C_{ijk} = 0$, then this term is set equal to 0.

4) Calculate for each row (set of parallel plates) the $G_{(2)}^2$ value (i.e. G^2 with 2 degrees of freedom if no data are missing) by summing the values for the three plates and multiplying the sum by two:

$$G_{(2)}^2 = 2\left[\sum_k^p \left(C_{ijk} \cdot \ln \frac{C_{ijk}}{E(C_{ijk})} \right)\right]$$

Note this value down in the table.

5) Sum those $G_{(2)}^2$ values over all the series and dilutions in the table. In this way, the following test value is obtained:

$$G_A^2 = \sum_i^s \sum_j^d G_{(2)}^2$$

Compare the value of G_A^2 with the tabulated values of χ^2 (chi-squared) in Table 5, where df equals the number of counts used in the calculations minus 1. If the value of G_A^2 is *less* than the tabulated 0,01 probability value, then the counts are statistically homogeneous. All steps in the performance of the method (sub-sampling, diluting, plating, counting) may be considered acceptable. No further evaluation is necessary and the analysis may be concluded here.

Table 5 — Values for χ^2 (P = 0,01)

df	$\chi^2_{0,01}$	df	$\chi^2_{0,01}$
56	83,53	67	96,82
57	84,75	68	98,02
58	85,96	69	99,22
59	87,17	70	100,42
		71	101,62

If the value of G_A^2 is *greater* than the tabulated 0,01 probability value, then perform an analysis of variance (ANOVA) on the data in order to identify and to quantify the sources of the excessive variability (sub-sampling, diluting and plating/counting).

There are two possible reasons for a high value of G_A^2.

a) When the excess is moderate, the reason is probably that there are a few steps in the method which show a somewhat larger variability than would be expected if the method were applied correctly. However, this excessive variability is not so large that the results cannot be used in practice. In this case, a G_A^2 function is a warning sign: the method may still be applied in this way, but regular control is necessary to make sure that the variability does not worsen.

b) When the excess is considerable (G_A^2 much greater than the P = 0,01 value), one or more steps have an unacceptably high variability.

Scrutinize and correct factors with a too high variability.

EXAMPLE Four dilution series, S_1, S_2, S_3 and S_4, were taken from a well-homogenized and appropriately diluted sample. From each sub-sample, a binary dilution series was laid out and from each dilution D_j, three parallel plates, P_1, P_2 and P_3, were inoculated. Six dilutions provided countable results. The counts are presented in Table 6. All values have been calculated with the full power of the calculator, after which the results have been rounded to 2 or 3 decimal places.

In Table 6, column C_{ij} contains the means of the three parallel plates; column $E(C_{ijk})$ contains the expected means for the dilutions.

The values for $E(C_{ijk})$ are calculated as follows (dilution steps used: $D_1 = 2^{-6}$ to $D_6 = 2^{-11}$).

Volumes are expressed in relative units (the highest dilution 2^{-11} is taken as the unit volume):

— V_1 (corresponding to D_1) = $2^5 V_6$ = 32

— V_2 (corresponding to D_2) = $2^4 V_6$ = 16

— V_3 (corresponding to D_3) = $2^3 V_6$ = 8

— V_4 (corresponding to D_4) = $2^2 V_6$ = 4

— V_5 (corresponding to D_5) = $2^1 V_6$ = 2

— V_6 (corresponding to D_6) = 1

— Total number of colonies: 84 + 113 + 109 + 74 + … + 1 + 7 + 4 = 4862

— Total volume (in units of V_6): (12 × 32) + (12 × 16) + (12 × 8) + (12 × 4) + (12 × 2) + (12 × 1) = 756

— Expected number of counts $E(C_{ijk})$ for a volume V_6 = 4862/756 = 6,43

— Expected number of counts $E(C_{ijk})$ for a volume V_5 = 4862/756 x 2 = 12,86

— Expected number of counts $E(C_{ijk})$ for a volume V_4 = 4862/756 x 4 = 25,72

— Expected number of counts $E(C_{ijk})$ for a volume V_3 = 4862/756 x 8 = 51,45

— Expected number of counts $E(C_{ijk})$ for a volume V_2 = 4862/756 x 16 = 102,90

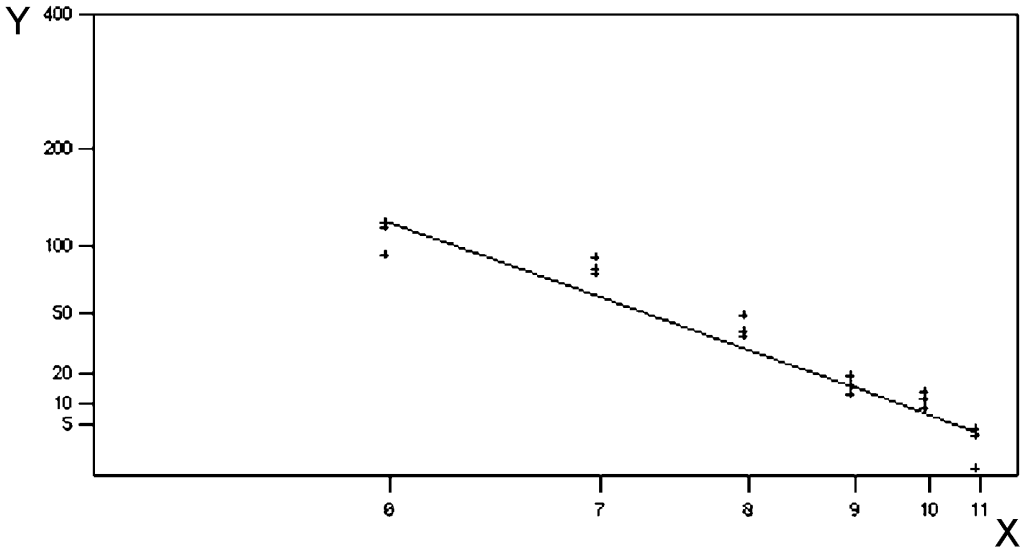
— Expected number of counts $E(C_{ijk})$ for a volume V_1 = 4862/756 x 32 = 205,80

Table 6 — Original data

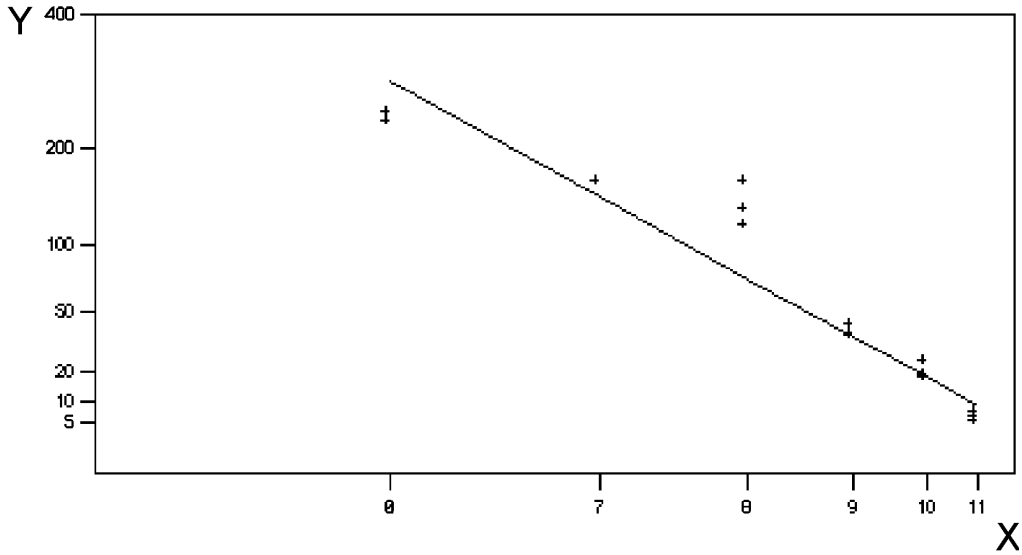
$\mathbf{S}_i$	$\mathbf{D}_j$		$\mathbf{P}_k$			C_{ij}	$E(C_{ijk})$	G^2
			$\mathbf{P}_1$	$\mathbf{P}_2$	$\mathbf{P}_3$			
S_1	D_1	2^{-6}	84	113	109	102,00	205,80	4,997
	D_2	2^{-7}	74	82	70	75,33	102,90	0,984
	D_3	2^{-8}	35	43	33	37,00	51,45	1,483
	D_4	2^{-9}	10	13	16	13,00	25,72	1,397
	D_5	2^{-10}	7	11	9	9,00	12,86	0,896
	D_6	2^{-11}	0	2	3	1,67	6,43	4,256
S_2	D_1	2^{-6}	238	236	226	233,33	205,80	0,356
	D_2	2^{-7}	154	153	153	153,33	102,90	0,004
	D_3	2^{-8}	154	126	111	130,33	51,45	7,226
	D_4	2^{-9}	33	34	38	35,00	25,72	0,395
	D_5	2^{-10}	16	15	21	17,33	12,86	1,161
	D_6	2^{-11}	4	5	6	5,00	6,43	0,403
S_3	D_1	2^{-6}	154	151	136	147,00	205,80	1,280
	D_2	2^{-7}	84	68	72	74,67	102,90	1,831
	D_3	2^{-8}	44	65	63	57,33	51,45	4,899
	D_4	2^{-9}	25	35	25	28,33	25,72	2,275
	D_5	2^{-10}	13	13	13	13,00	12,86	0,000
	D_6	2^{-11}	5	0	3	2,67	6,43	6,993
S_4	D_1	2^{-6}	238	236	224	232,67	205,80	0,496
	D_2	2^{-7}	154	156	146	152,00	102,90	0,371
	D_3	2^{-8}	63	61	56	60,00	51,45	0,437
	D_4	2^{-9}	17	32	28	25,67	25,72	4,980
	D_5	2^{-10}	11	10	12	11,00	12,86	0,182
	D_6	2^{-11}	1	7	4	4,00	6,43	5,062
								Sum = G_P^2 = 52,364
Indices: i = 4, j = 6, k = 3								

The data for each dilution series are shown graphically in Figure 3. The straight line in each of the four figures indicates what would be expected if the dilutions within each series were made perfectly. Each value within a dilution series is represented by the symbol "+".

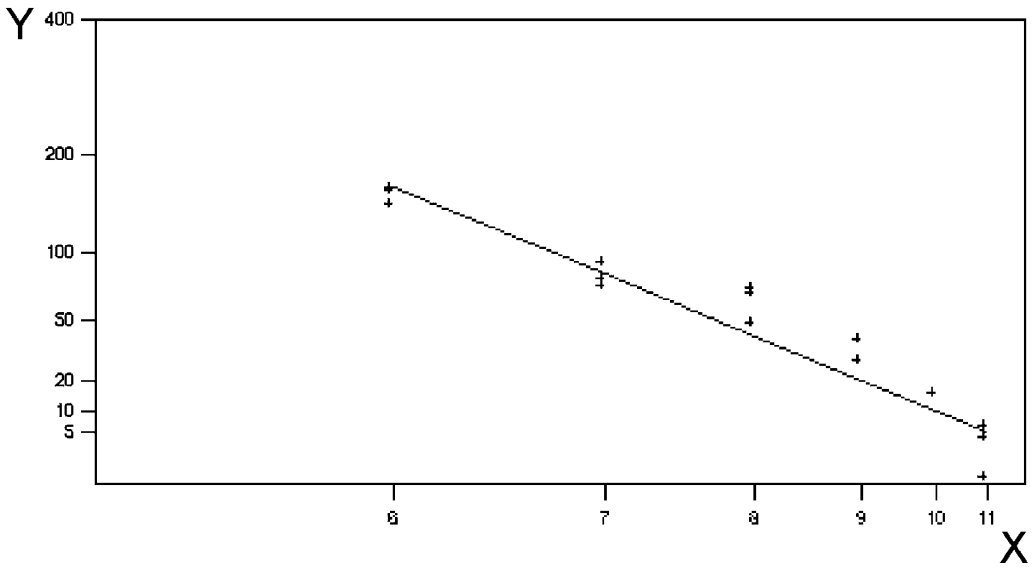
Figure 4 a) shows the four straight lines of Figure 3 in the same graph. Their mutual deviations give an indication of the quality of the sub-sampling. Figure 4 b) shows the means of the parallel plates (column C_{ij} of Table 6) for each series. In the ideal case, all points should fall on the same line.



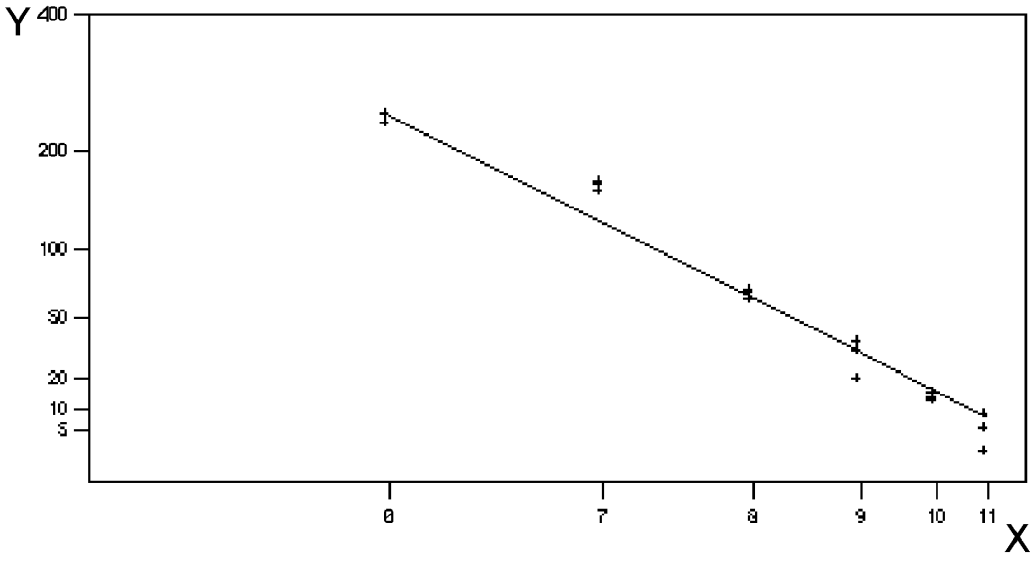
a) Dilution series 1



b) Dilution series 2



c) Dilution series 3



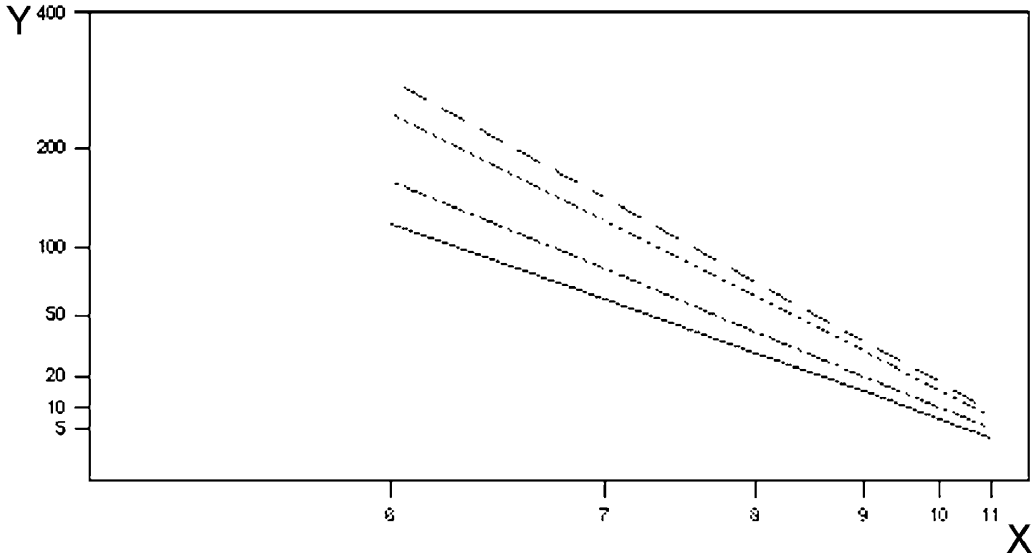
d) Dilution series 4

Key

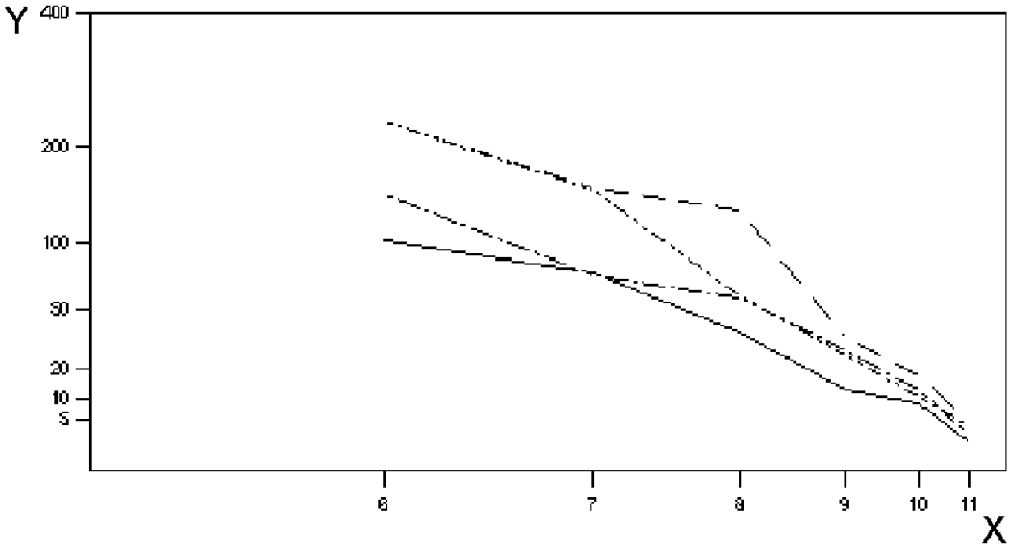
X dilution 2^{-x} (square root scale)

Y colony count (square root scale)

Figure 3 — Dilution series



a) Weighted averages for each series



b) Parallel plate averages for each series

Key

X dilution 2^{-x} (square root scale)

Y colony count (square root scale)

Figure 4 — Weighted averages and parallel plate averages

10.2.3.2 Calculation of the test value $G_P{}^2$

For this calculation, the values in the column C_{ij} are used.

Calculate for each row of parallel plates the $G_{(2)}{}^2$ value (last column of Table 6):

2 [84·ln (84/102) + 113·ln (113/102) + 109·ln (109/102)] = 4,997

2 [74·ln (74/75,33) + 82·ln (82/75,33) + 70·ln (70/75,33)] = 0,984

…

2 [1·ln (1/4) + 7·ln (7/4) + 4·ln (4/4)] = 5,062

Add all the values to obtain the overall measure of the homogeneity of the parallel plating:

G_P^2 = 4,997 + 0,984 + … + 5,062 = 52,364.

The number of degrees of freedom of G_P^2 is found by adding the degrees of freedom of all the G^2. In the example no counts are missing, so each row has 2 degrees of freedom and the total df = 48.

The value of G_P^2 (52,364) is greater than the value 26,51 and clearly smaller than the value 73,68 in Table 4 and therefore the variability between the parallel plates is well within acceptable limits.

10.2.3.3 Calculation of the test value G_A^2

Here the values in the column $E(C_{ijk})$ are used in the calculation:

G_A^2 = 2 [84·ln (84/205,80) + 113·ln (113/205,80) + … + 4·ln (4/6,43)]

= 2 (420,35) = 840,70

Degrees of freedom = (72 – 1) = 71

The value of G_A^2 (840,70) is much larger than the value of 101,62 in Table 5. Therefore the overall variability is excessive; the statistical analysis should be continued to investigate the source(s) of this large variability.

10.2.4 Where are the errors? Analysis of variance (see Figure 2)

10.2.4.1 With each factor in the procedure, a certain variability (variance in statistical terminology) is associated. Apart from the ideal variability between parallel plates, it is not known how large the variance of each factor will be if the method is applied correctly. However, a variance much larger than the expected value indicates problems with that particular step of the method.

As each result is subject to several sources of variation (sub-sampling/diluting/plating/possible interactions between those factors), it is necessary to extract the variation associated with each source from the total data. This is done using a statistical technique known as “analysis of variance”.

NOTE 1 For the theoretical background of this technique, see any handbook on statistics.

NOTE 2 If data are missing (see 10.1), the corresponding values can be estimated by taking the mean of the parallel plate counts of the same series and the same dilution. The error introduced by this procedure is small and has a negligible effect on the conclusions.

10.2.4.2 The extraction of the variances is performed as follows.

The tabulated counts C_{ijk} (see 10.2.2) are transformed into the values T_{ijk} using the formula

$$T_{ijk} = \sqrt{C_{ijk}} - \sqrt{E(C_{ijk})}$$

where $E(C_{ijk})$ is the expected value for each dilution (see 10.2.3).

NOTE If one or more missing data have been estimated (see Note 2 in 10.2.4.1), then before the transformation the expected values $E(C_{ijk})$ of the whole data set have to be recalculated first.

Microbiological counts follow a statistical distribution known as the Poisson distribution. The analysis of variance, however, assumes a Gaussian distribution (also known as a “normal distribution”) of the data, therefore the raw data cannot be used directly in the calculations. The square-root transformation of the data brings the variance of the counts more in accordance with the theoretical assumptions of the analysis of variance.

10.2.4.3 From the transformed data, T_{ijk}, a number of sums are calculated.

The sum of all transformed values:

$$(v) = \sum_{i}^{s} \sum_{j}^{d} \sum_{k}^{p} T_{ijk}$$

where

s is the number of dilution series S_i (= 4);

d is the number of dilution steps D_j (= 5 or 6);

p is the number of plates P_k (= 3).

The sum of the squares of all transformed values:

$$(w) = \sum_{i}^{s} \sum_{j}^{d} \sum_{k}^{p} T_{ijk}^{2}$$

The sum of the squares of the totals of each group of replicate plates, P_k

$$(x) = \sum_{i}^{s} \sum_{j}^{d} \left[\sum_{k}^{p} T_{ijk} \right]^2$$

The sum of the squares of the totals of the dilution series S_i

$$(y) = \sum_{i}^{s} \left[\sum_{j}^{d} \sum_{k}^{p} T_{ijk} \right]^2$$

The sum of the squares of the totals of the dilutions D_j

$$(z) = \sum_{j}^{d} \left[\sum_{i}^{s} \sum_{k}^{p} T_{ijk} \right]^2$$

10.2.4.4 These sums are used in the calculation of the sums of squares:

Source of variation	**Sum of squares**
Between dilution series S	$(\Sigma 1) = \dfrac{s \times (y) - (v)^2}{s \times d \times p}$
Dilution steps D within dilution series S	$(\Sigma 2) = \dfrac{d \times (x) - (y)}{d \times p}$
Between parallel plates	$(\Sigma 3) = (w) - \dfrac{(x)}{p}$
Total	$(\Sigma 4) = (w) - \dfrac{(v)^2}{s \times d \times p}$

The sums of squares are used in the calculation of the mean squares, which in their turn are used, together with the mean squares as expected from theory, in the determination of the variances. These steps can be presented as in Table 7.

Table 7 — ANOVA: mean squares and expected mean squares

Source of variation	Sum of squares	df	Mean squares	Expected mean square
Between dilution series S	(Σ1)	$s - 1$	$s_1^2 = \frac{(\Sigma 1)}{s-1}$	$\sigma_p^2 + p\sigma_d^2 + dp\sigma_s^2$
Between dilution steps D within series	(Σ2)	$s(d - 1)$	$s_2^2 = \frac{(\Sigma 2)}{s(d-1)}$	$\sigma_p^2 + p\sigma_d^2$
Between parallel plates	(Σ3)	$s \times d\ (p - 1)$	$s_3^2 = \frac{(\Sigma 3)}{s \times d(p-1)}$	σ_p^2
Total	(Σ4)	$s \times d \times p - 1$		

The variance between plates, σ_p^2, is estimated by the term s_3^2. The variance between dilution steps within dilution series σ_d^2 is estimated by the term $(s_2^2 - s_3^2)/p$ and the variance between the dilution series s_s^2 is estimated by the term $(s_1^2 - s_2^2)/dp$.

The total variance $\sigma_T^2 = \sigma_p^2 + \sigma_d^2 + \sigma_s^2$.

10.2.5 Evaluation of the variances

The ideal value for σ_p^2 should be about 0,25; experience has shown that a total variance σ_T^2 of more than 1 is indicative of serious trouble.

If the total variance σ_T^2 is *less* than 1, then the method is likely to be under statistical control. If the total variance σ_T^2 is *greater* than 1, then one or more factors are not under statistical control. In this case, the analysis of variance shall be extended in order to locate the weak points in the performance of the method.

This extension of the statistical analysis consists of two steps. First, the variance associated with the “dilution steps within series” is divided into a variance associated with the dilution steps and a variance due to the possible interaction between dilution steps and dilution series. Thereafter the variances are tested for statistical significance, which allows rating of the factors in order of importance. The following supplementary values should be calculated first.

Sum of squares between dilutions D:

$$(\Sigma 5) = \frac{d(z) - (v)^2}{s \times d \times p}$$

Sum of squares of the interaction is equal to: (Σ6) = (Σ2) – (Σ5)

The results for the complete analysis of variance are presented in Table 8.

Table 8 — Results of analysis of variance

Source of variation	Sum of squares	df	Mean squares	F-value
Between dilution series S	(Σ1)	$s - 1$	$s_1^2 = \frac{(\Sigma 1)}{s-1}$	$\frac{s_1^2}{s_6^2}$
Between dilution steps D	(Σ5)	$d - 1$	$s_5^2 = \frac{(\Sigma 5)}{d-1}$	$\frac{s_5^2}{s_6^2}$
Interaction	(Σ6)	$(s-1)(d-1)$	$s_6^2 = \frac{(\Sigma 6)}{(s-1)(d-1)}$	$\frac{s_6^2}{s_3^2}$
Between parallel plates	(Σ3)	$s \times d(p-1)$	$s_3^2 = \frac{(\Sigma 3)}{s \times d(p-1)}$	
Total	(Σ4)	$(s \times d \times p) - 1$		

For testing the significance of the variances, the corresponding F-values are determined. F-values are the ratios of two variances, where the numerator is the calculated variance associated with the factor under investigation, and the denominator is the variance expected if the same factor did not contribute to the overall variance. If the factor under investigation has no effect, then the two variances are independent estimates of the same values and their ratio should fall within a predefined range. If the factor is associated with an important increase in variability however, then the variance in the numerator becomes so large that the F-ratio falls outside this predefined range.

The F-values obtained are compared with the corresponding entries $F(f1, f2)$ in Table 9 for a probability level of P = 0,01. The symbols $f1$ and $f2$ are the number of degrees of freedom (column df in Table 8) of the variance in the numerator and the variance in the denominator respectively.

Table 9 — F-values for selected combinations of $f1/f2$ (P = 0,01)

$f1$	$f2$	F
3	12	5,95
3	15	5,42
4	12	5,41
5	15	4,56
12	40	2,66
15	48	2,44

10.2.6 Conclusions and further action

Factors for which the F-value exceeds the critical value in Table 9 should be investigated thoroughly. They show aspects (sample homogeneity, manipulation) that should be brought under control before the colony count can be applied further. The interpretations to be given to a significant value for the different factors are as follows.

a) between dilution series S: a systematic error in the preparation of the different dilution series (homogenization of the sample material, dispensing);

b) between dilution steps D: an error in the way the dilution steps are prepared;

c) interaction: a general error in the performance of the work;

d) between plates: if the between-plates variance is much greater than 0,25, an explanation shall be sought; if the high value is not due to some excessively variable parallel sets (see the components of the G_P^2 value in Table 6) then investigate the stacking effect by summing the colony counts of the data according to their position in the stack (top-middle-bottom);

e) evaluate also the effect of a high residual variance on the analysis of variance.

After investigation and correction of the deficient steps in the application of the method, the entire procedure shall be repeated in order to confirm that the problems have been corrected.

EXAMPLE The data of Table 6 are replaced by the transformed values; in the two columns on the right the sum of the values of each row and the sum of the squared values of each row are given.

Table 10 — Transformed data

S_i	D_j			P_k		Sum	Sum of squares
			P_1	P_2	P_3		
S_1	D_1	2^{-6}	−5,181	−3,716	−3,905	−12,801	55,895
	D_2	2^{-7}	−1,542	−1,089	−1,777	−4,408	6,720
	D_3	2^{-8}	−1,257	−0,615	−1,428	−3,300	3,998
	D_4	2^{-9}	−1,910	−1,466	−1,072	−4,448	6,946
	D_5	2^{-10}	−0,941	−0,270	−0,586	−1,797	1,302
	D_6	2^{-11}	−2,536	−1,122	−0,804	−4,462	8,336
S_2	D_1	2^{-6}	1,082	1,017	0,688	2,786	2,676
	D_2	2^{-7}	2,266	2,225	2,225	6,716	15,038
	D_3	2^{-8}	5,237	4,052	3,363	12,652	55,153
	D_4	2^{-9}	0,673	0,759	1,092	2,524	2,222
	D_5	2^{-10}	0,414	0,287	0,996	1,696	1,245
	D_6	2^{-11}	−0,536	−0,300	−0,086	−0,922	0,385
S_3	D_1	2^{-6}	−1,936	−2,057	−2,684	−6,677	15,184
	D_2	2^{-7}	−0,979	−1,898	−1,659	−4,535	7,311
	D_3	2^{-8}	−0,540	0,889	0,764	1,114	1,667
	D_4	2^{-9}	−0,072	0,844	−0,072	0,700	0,723
	D_5	2^{-10}	0,019	0,019	0,019	0,057	0,001
	D_6	2^{-11}	−0,300	−2,536	−0,804	−3,640	7,167
S_4	D_1	2^{-6}	1,082	1,017	0,621	2,719	2,589
	D_2	2^{-7}	2,266	2,346	1,939	6,551	14,398
	D_3	2^{-8}	0,764	0,637	0,310	1,712	1,087
	D_4	2^{-9}	−0,949	0,585	0,220	−0,144	1,291
	D_5	2^{-10}	−0,270	−0,424	−0,122	−0,816	0,268
	D_6	2^{-11}	−1,536	0,110	−0,536	−1,962	2,659
Total sum (v)						−10,685	
Total sum of squares (w)							214,260

(x) = the sum of [the squares of the totals for each group of replicate plates]
= the sum of the squares of the values in the column "Sum" of Table 10
= $(-12{,}801)^2 + (-4{,}408)^2 + ... + (-1{,}962)^2$
= 598,070

(z) = the sum of [the squares of the totals per dilution series]

Dilution series	Total	Squares
S_1	–31,216	974,445
S_2	25,452	647,800
S_3	–12,981	168,494
S_4	8,059	64,953
Total	(v)	1 855,693 (y)

The sums (v), (w), (x) and (y) are used in the calculation of the sums of squares:

Source of variation	Sum of squares
Between dilution series *S*	$(\Sigma 1) = \frac{4(1\,855{,}693) - (-10{,}685)^2}{4 \times 6 \times 3} = 101{,}508$
Dilution steps D within dilution series S	$(\Sigma 2) = \frac{6(598{,}070) - (1\,855{,}693}{6 \times 3} = 96{,}263$
Between parallel plates	$(\Sigma 3) = 214{,}260 - \frac{598{,}070}{3} = 14{,}903$
Total	$(\Sigma 4) = 214{,}260 - \frac{(-10{,}685)^2}{4 \times 6 \times 3} = 212{,}674$

With the sums of squares obtained, the mean squares are calculated after which, by equating them with the mean squares as expected from theory, leads to the estimation of the variances.

Source of variation	Sum of squares	df	Mean squares
Between dilution series S	($\Sigma 1$)	3	$s_1^2 = \frac{101{,}505}{3} = 33{,}836$
Dilution steps D within series	($\Sigma 2$)	20	$s_2^2 = \frac{96{,}263}{20} = 4{,}813$
Between parallel plates	($\Sigma 3$)	48	$s_3^2 = \frac{14{,}903}{48} = 0{,}310$

The estimate for the variance components (see Table 7) are:

σ_p^2 is estimated by s_3^2 = 0,310;

σ_d^2 is estimated by $(s_2^2 - s_3^2)/p$ = (4,813 – 0,310)/3 = 1,501;

σ_s^2 is estimated by $(s_1^2 - s_2^2)/dp$ = (33,836 – 4,813)/18 = 1,612;

σ_T^2 is estimated as 0,310 + 1,501 + 1,612 = 3,424.

As this last value is greater than the maximum allowable value of 1, a further investigation of the sources of the errors shall be conducted.

The following values have to be calculated first:

(z) = the sum of the squares of the totals per dilution step

Dilution steps	Sum	Squares
D_1	–13,974	195,271
D_2	4,325	18,703
D_3	12,178	148,299
D_4	–1,368	1,872
D_5	–0,859	0,739
D_6	–10,986	120,695
Total	(v)	485,579 (z)

Sum of squares for the dilution steps $(\Sigma 5) = \dfrac{6(485{,}579) - (-10{,}685)^2}{4 \times 6 \times 3} = 38{,}879$

Sum of squares of the interaction ($\Sigma 6$) = 96,263 – 38,879
= 57,384

Using these values, the table for the analysis of variance (see Table 8) can be completed:

Table 11 — Calculation

Source of variation	Sum of squares	df	Mean squares	F-value
Between dilution steps S	101,508	3	s_1^2 = 33,836	8,845[a]
Between dilution steps D	38,879	5	s_5^2 = 7,776	2,033 (n.s.)[b]
Interaction	57,384	15	s_6^2 = 3,826	12,321[a]
Between parallel plates	14,903	48	s_3^2 = 0,310	
Total	212,674	71		

[a] Significant at the P = 0,01 significance level.

[b] n.s. means not significant.

Comparison of the tabulated values for F for a probability of 0,01 (Table 9) with the calculated values results in the following conclusions:

— between dilution series *S*: $F_{0,01}$ (3;15) = 5,42 < 8,845 significant;

— between dilution steps D: $F_{0,01}$ (5;15) = 4,56 > 2,033 not significant;

— interaction: $F_{0,01}$ (15;48) = 2,44 < 12,321 significant.

The high value for “between dilution series S” indicates a systematic error in the preparation of the dilution series (homogenization of the sample material, dispensing), or instability of the population.

The high value for “interaction” indicates statistically that the linearity (or lack of linearity) of the colony counts differs among the four dilution series (see Figures 3 and 4). This is a strong indication of errors in the technical performance, but may also indicate biological interactions or growth anomalies that the person counting the colonies is not familiar with.

The acceptable value for “between dilution steps D” in this case, where the interaction is significant, is far from informative. The result only shows that no systematic general error is shown in the way the dilutions are made.

Annex A
(informative)

Weighted mean and homogeneity testing of colony counts

A.1 Introduction

When countable numbers of colonies are available from parallel plates, from different volumes of a suspension, or from more than one dilution; it is reasonable to try to use all the information to obtain the best possible density estimate. Computing the weighted mean does this. The principle was probably first published in microbiological literature (see Reference [2]).

It is often stated that only colony count numbers between 30 and 300 or between 25 and 250 are “reliable”. If these rules are followed, there usually remains only one dilution to count colonies from, and there is no need to calculate the weighted mean. It seems a waste, however, to ignore the information contained in plates with less than 25 colonies, especially because plates with low colony numbers are biologically the most reliable. A lower limit of about five colonies per plate on average would be a suitable lower limit for statistical reasons.

The idea of the weighted mean is to divide the sum of all observed colony counts by the sum of all volumes involved (expressed in term of the original sample). The most general expression of the principle is therefore:

$$M = \frac{\sum C_i}{\sum V_i} = \frac{C_1 + C_2 + ... + C_n}{V_1 + V_2 + ... + V_n} \qquad \text{(A.1)}$$

where

M is the weighted mean colony count per millilitre of original sample;

C_i are the number of colonies counted on the ith plate;

V_i is the volume used to count the colonies on the ith plate;

i is the number of the plate used (i = 1, 2,.... n);

n is the total number of plates used.

In order to pool the data with sufficient confidence, it is first necessary to test that the series of colony numbers is homogeneous (random variation). To do this, a general homogeneity index is needed to test whether all the observed colony counts could have arisen in the case where the exact volumes V_1, V_2, ..., V_n were sampled from one homogeneous suspension. The best available test, G^2, is described in A.2.

If the test proves that the data are inhomogeneous, one should not use all the counts for computing the mean. At the same time, the result would be a signal to investigate further the reasons for this inhomogeneity.

Two main uses are made of the G^2 homogeneity index in the colony count context. It is used to confirm the homogeneity of the data before calculating the weighted mean, and it can be applied in the so-called “analysis of deviance” to find the causes of inhomogeneity in colony count data. In this part of ISO 14461|IDF 169, the G^2 index is used for the general homogeneity test and, in addition, to test the overall homogeneity of parallel plating.

A.2 The G^2-test of randomness (homogeneity)

A homogeneity index well suited to testing colony count data is the likelihood ratio index G^2. It is most simply expressed as:

$$G^2_{n-1} = 2\left[\sum_{i=1}^{n} O_i \ln\left(\frac{O_i}{E_i}\right)\right] \tag{A.2}$$

where

O_i is the ith observed frequency (count);

E_i is the ith expected frequency (count);

i is the number of the frequency (i = 1, 2,… n);

n is the total number of frequencies.

In testing the "internal homogeneity", the assumption is made that the observed counts should follow the volumes studied. Therefore, the expected counts E_i are obtained by calculating the fraction of the sum of all observed colonies that each of the volumes should theoretically contain:

$$E_i = \frac{V_i}{\sum V_i}\sum C_i \tag{A.3}$$

In practice, the general formula (A.2) involves the calculation of each E_i value and is not needed for other purposes (in the main text Equation (A.2) is used as the E_i values are needed in the ANOVA). In that case, inserting the expected frequencies from Equation (A.3) and reworking the formula, a more useful form is obtained:

$$G^2_{n-1} = 2\left[\sum C_i \cdot \ln\frac{C_i}{V_i} - \left(\sum C_i\right)\cdot \ln\left(\frac{\sum C_i}{\sum V_i}\right)\right] \tag{A.4}$$

It is obvious that in internal homogeneity testing, the actual volume and any numbers proportional to them can replace V_i. In most cases, the scale can be selected in such a way that these relative volumes R_i become simple integers. This change makes the formula quite simple to use with hand-held calculators:

$$G^2_{n-1} = 2\left[\sum C_i \cdot \ln\frac{C_i}{R_i} - \left(\sum C_i\right)\cdot \ln\left(\frac{\sum C_i}{\sum R_i}\right)\right] \tag{A.5}$$

where

C_i is the number of colonies counted on the ith plate;

V_i is the real volume used on the ith plate;

R_i is the corresponding relative volume used on the ith plate;

i is the number of the plate used (i = 1, 2,…, n);

n is the total number of plates used.

High index values indicate variation that is higher than random, i.e. so-called *over-dispersion*. Very low index values indicate variation that is unnaturally low (*under-disperion*).

The statistical significance of over- or under-dispersion can be judged by comparing the calculated values with the theoretical χ^2 distribution at the selected probability with $n - 1$ degrees of freedom. Values needed in the examples that follow are reproduced in Table A1. Calculations outlined above give the general homogeneity index G_A^2 used in the main text.

A BASIC programme for calculating the index according to Equation (A.5) is given in Annex B.

Table A.1 — Selected critical values of the χ^2 distribution

df	Probability					
	Under-dispersion			Over-dispersion		
	0,99	0,95	0,10	0,05	0,01	0,001
1	—	0,004	2,71	3,84	6,63	10,38
2	0,020	0,103	4,61	5,99	9,21	13,81
3	0,115	0,352	6,25	7,81	11,34	16,27
4	0,297	0,711	7,78	9,49	13,28	18,47
5	0,554	1,145	9,24	11,07	15,09	20,52

A.3 Overall agreement on parallel plating

The second use made of the G^2 test in this part of ISO 14461|IDF 169 is to examine the homogeneity of parallel plating. Calculating the value of the homogeneity index for each set of parallel plates can do this. The task is simplified by the fact that all the volumes (or relative volumes) in the parallel set are equal and may be replaced by the value 1. The sum of the relative volumes thus becomes equal to the number of parallel plates (n) and Equation (A.5) can be rewritten as:

$$G_{n-1}^2 = 2\left[\sum C_i \cdot \ln C_i - \left(\sum C_i\right) \cdot \ln\left(\frac{\sum C_i}{n}\right)\right] \tag{A.6}$$

where

C_i is the number of colonies counted on the ith plate;

i is the number of the plate used (i = 1, 2,...., n);

n is the number of parallel plates.

A solitary value of the homogeneity index is of rather limited use. If, however, there are m sets of plates with equal numbers of parallels, then we can take advantage of the additive property of G^2. By adding the G^2 values and their corresponding degrees of freedom from all sets, an overall measure of homogeneity is obtained. This is called G_P^2 in this part of ISO 14461|IDF 169:

$$G_P^{\ 2} = G_{m(n-1)}^2 = \sum_{j=1}^{m} G_{(n-1)}^2 \tag{A.7}$$

Some over-dispersion must be tolerated in microbiological work, because volume measurements, still conforming to acceptable working standards, are never absolute accurate. It seems, therefore, that the 1 % (P = 0,01) probability level instead of the usual 5 % level should be taken as the "*warning limit*".

A.4 Examples

A.4.1.1 Example 1: General homogeneity test

A set of colony counts has been obtained from two dilutions that differ by a factor of 10. Two parallel series of plates have been made at each dilution. The counts are given in Table A.2, together with the relative volumes.

Table A.2 — Two parallel series counts and relative volumes

Dilution	Colony counts		Sum	Relative volume		Sum
10^{-4}	251	305	556	10	10	20
10^{-5}	31	36	67	1	1	2
Total			623			22

The randomness of the whole set as measured by the likelihood ratio index G^2 is calculated as follows:

$$G^2 = 2[251 \cdot \ln(251/10) + 305 \cdot \ln(305/10) + 31 \cdot \ln(31/1) + 36 \cdot \ln(36/1) - 623 \cdot \ln(623/22)]$$
$$= 7{,}607$$

As there are four terms in the sum, there are $4 - 1 = 3$ degrees of freedom. The calculated value shall be compared with the values on the third row of Table A1 of the χ^2 distribution.

The calculated value 7,607 indicates high variation but, compared with the theoretical distribution (1 % value = 7,81), the data set as a whole can be considered a random sample from a homogeneous suspension. The ratio of counts between the dilutions (556/67 = 8,3) is rather different from the ideal value of 10:1, but might have arisen by chance. There is no sufficient statistical reason to consider the data inhomogeneous. The weighted mean can therefore be calculated according to Equation (A.1) using all available colony counts.

A.4.1.2 Example 2: General homogeneity test followed by analysis of deviance

Another similar data set, but with three parallels, had the values shown in Table A.3.

Table A.3 — Three parallel series counts and relative volumes

Dilution	Colony counts			Sum	Relative volume			Sum
10^{-5}	122	74	92	288	10	10	10	30
10^{-6}	12	15	10	37	1	1	1	3
Total				325				33

The data set appears to be quite over-dispersed, as shown by the index:

$$G^2 = 2[122 \cdot \ln(122/10 + 74 \cdot \ln(74/10) + \ldots + 10 \cdot \ln(10/1) - 325 \cdot \ln(325/33)]$$
$$= 15{,}077$$

This is almost the same as the 1 % value for 5 degrees of freedom. This result means that calculating the weighted mean based on the whole data set is unwise.

There seems to be good reason to examine the case further. Equation (A.3) can be used repeatedly to study the details of the data set.

The total variation consists of three components: the differences between the parallel plates of dilution 10^{-5}, the differences between the parallel plates of dilution 10^{-6} and the difference between the dilution levels:

$G_{(2)}^2$ = 2[122·ln(122/10) + 74·ln(74/10) + 92·ln(92/10) – 288·ln (288/30)]
= 12,127

$G_{(2)}^2$ = 2[12·ln 12 + 5·ln 15 + 10·ln 10 – 37·ln (37/3)]
= 1,020

$G_{(1)}^2$ = 2[288·ln(288/30) + 37·ln(37/3) – 325·ln (325/33)]
= 1,930

where the sub-indices of the G^2 denote the number of degrees of freedom attached to them.

This breakdown of the total variation forms an analysis of deviance and can be written as in Table A.4.

Note that the sum 12,127 + 1,020 + 1,930 = 15,077 is exactly equal to the total index calculated earlier.

Table A.4 — Total variation — Analysis of deviance

Source of deviation	G^2	df	P
Between dilutions	1,930	1	$> 0{,}10$
Parallel dilution 10^{-5}	1,020	2	$> 0{,}30$
Parallel dilution 10^{-6}	12,127	2	$> 0{,}01$
Total	15,077	5	$< 0{,}02$

When the calculated values are compared with the theoretical χ^2 distribution with the appropriate number of degrees of freedom, it appears that the significant cause of over-dispersion in the data is the inhomogeneity within the set of the parallel high colony (the lower dilution). These counts should not be utilized. This means that it is advisable to calculate the mean using only the set of low colony counts (the higher dilution). Thus, the data set would not be completely lost despite the general inhomogeneity of the original data.

A.4.1.3 Example 3: Homogeneity of parallel counts

The same person using two replicates has made five sets of colony counts. An assessment is to be made of the overall agreement of the parallel plates. The original counts, arranged in the order of increasing mean count, are shown in Table A.5. For each pair of counts, the homogeneity index is calculated using Equation (A.6).

Table A.5 — Pair of counts: Homogeneity index

Set No.	Parallel counts		G^2	df
1	22	18	0,401	1
2	35	41	0,474	1
3	80	99	2,021	1
4	191	164	2,056	1
5	340	297	2,905	1
Total			7,857	5

The sum (7,857) of the individual G^2 indices is the type of statistic applied in the main text to measure the overall agreement of parallel plating (G_P^2). According to the values shown on the fifth row of Table A.5, the overall fit of the parallels in the example seems satisfactory.

We can use these or similar data in other ways as well. We may compare each individual index with values of the χ^2 distribution with the corresponding degrees of freedom. However, isolated cases of bad fit or suspiciously perfect agreement should not be paid too much attention to, because G^2 is a random variable and may occasionally yield quite deviating values by chance.

When, however, tens or hundreds of index values are plotted in a control chart, the result may give useful information on not only the analyst performance but also on the limits of performance of the method.

When the data are obtained in the course of training, it may be natural to plot the individual index values in an historical order. Usually it is more informative to plot the values against the mean colony count.

There is often a trend towards high index values (worse fit, over-dispersion) as the mean count increases. Such a trend seems apparent in the example above. In the end, the results will indicate the upper (and possibly the lower) working limit of the method in the hands of the analyst in question (the index values should always be plotted on a personal control chart).

Annex B
(Informative)

BASIC programme for calculating the likelihood ratio index G^2

```
10    PRINT "LIKELIHOOD RATIO-INDEX G^2"
20    INPUT "NUMBER OF TERMS, n-"; N
30    C = 0; R = 0; S = 0; T = 0; D = 0
40    FOR I=1 TO N
50    PRINT "I ="; I
60    INPUT "COLONY COUNT="; C
70    INPUT "RELATIVE VOLUME="; R
80    IF (C = 0) THEN W = 0: GOTO 100
90    W = C*LOG(C/R)
91    REM IN THIS BASIC LOG = NATURAL LOGARITHM
100   S = S + W
110   T = T + R
120   D = D + C
130   NEXT I
140   Y = 2* (S - D*LOG (D/T)
150   REM IN THIS BASIC LOG = NATURAL LOGARITHM
160   PRINT
170   PRINT "INDEX G^2 ="; Y
180   PRINT
190   PRINT
200   INPUT "ANOTHER SET? (Y/N)"; H$
210   IF H$ = Y" GOTO 20, ELSE TO 220
220   END
```

Bibliography

[1] ISO 707 | IDF 50, *Milk and milk products — Guidance on sampling*

[2] FARMILOE, F.J., CORNFORD, S.J., COPPOCK, J.B.M. and INGRAM, M. The survival of *Bacillus subtilis* spores in the baking of bread. *J. Sci. Food. Agric*., **5**, 1954, pp. 294-404

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