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MICROCRM: Feasibility certification studies of microbiological reference materials

K.A. Mooijman, N.J.D. Nagelkerke, C. Demarquilly[1], M. Lemdani[1], D. Stewardson[2], T. Fouweather[2], N. Lightfoot[3], T. Simonart[4]

(1) University of Lille II, France
(2) University of Newcastle, United Kingdom
(3) Health Protection Agency (HPA), Newcastle, United Kingdom
(4) Institut Pasteur de Lille, France (co-ordinaror)

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RIVM, P.O. Box 1, 3720 BA Bilthoven, telephone: 31 - 30 - 274 91 11; telefax: 31 - 30 - 274 29 71

Abstract

In 2002 feasibility certification studies were carried out on three different types of microbiological reference materials (RMs) for eight different ISO and EN standard methods. These studies were performed as part of the European project ‘Microbiological Certified Reference Materials in support of EU water legislation, performance testing and laboratory quality control’ (MICROCRM 01/02/2001 – 01/11/2003). The aim of the project was to determine the conditions necessary to produce and certify key reference materials for water microbiology. The three different types of RMs were capsules, lenticules and pastilles. ISO and EN standard methods related to EU water legislation were used (for the Drinking-water Directive and the Bathing-water Directive). For each type of RM, eight batches - containing different strains - were prepared (for use for the eight different methods). Thirteen European laboratories participated in the studies. The results of the studies were statistically analysed by three statisticians of the three partners in the project. The main conclusion was that certification of the microbiological RMs was feasible for all target parameters at the desired concentration levels for the two directives mentioned above.

Rapport-in-het-kort

In 2002 werden haalbaarheid certificeringsringonderzoeken georganiseerd met drie verschillende typen microbiologische referentiematerialen voor acht verschillende ISO en EN standaard methoden, gerelateerd aan EU water wetgeving (Drinkwaterrichtlijn en Zwemwaterrichtlijn). De studies werden uitgevoerd in het kader van het Europese project: ‘Microbiologische gecertificeerde referentiematerialen ter ondersteuning van EU water wetgeving, testen van performance en laboratorium kwaliteitscontrole’ (MICROCRM 01/02/2001 – 01/11/2003). De doelstelling van het MICROCRM project was om de condities te bepalen welke nodig zijn voor de productie en certificering van belangrijke referentiematerialen voor watermicrobiologie. De drie verschillende typen referentiematerialen waren capsules, lenticules en pastilles. Voor ieder type referentiemateriaal werden acht partijen, met verschillende stammen, bereid (om te gebruiken met de acht verschillende methoden). Dertien Europese laboratoria namen deel aan de studies. De resultaten van de studies werden statistisch geanalyseerd door drie statistici van de drie partners in het project. De belangrijkste conclusie was dat certificering van de microbiologische referentiematerialen haalbaar was voor alle tot doel gestelde parameters op de gewenste besmettingsniveaus voor de richtlijnen zoals hierboven vermeld.

Contents

Abbreviations and symbols

BW	Bathing water
cfp	colony forming particles
CRM	Certified Reference Material
df	degrees of freedom
EN	European Standard
DW	Drinking water
IPL	Institut Pasteur de Lille
ISO	International Organization for Standardization
PHLS	Public Health Laboratory Service
RIVM	National Institute for Public Health and the Environment
RM	Reference Material

Samenvatting

Het Europese project ‘MICROCRM’ startte op 1 februari 2001 en duurde tot 1 november 2003. De afkorting ‘MICROCRM’ staat voor: ‘Microbiologische gecertificeerde referentie-materialen ter ondersteuning van EU water wetgeving, testen van performance en laboratorium kwaliteitscontrole’. De doelstelling van het MICROCRM project was om de condities te bepalen welke nodig zijn voor de productie en certificering van microbiologische referentiematerialen voor ondersteuning van EU water wetgeving (Drinkwaterrichtlijn en Zwemwaterrichtlijn). Haalbaarheid certificeringsstudies werden uitgevoerd in 2002. Hiervoor produceerden de drie partners van het project partijen van één van de volgende drie typen referentiematerialen (RMs): capsules, lenticules, pastilles. Van ieder type RM werden acht partijen, met verschillende bacteriologische stammen, bereid. Alle partijen RMs werden gecontroleerd op gemiddeld besmettingsniveau, homogeniteit en stabiliteit. Een vast aantal van alle typen en partijen RMs werden naar dertien (vooraf geselecteerde en getrainde) Europese laboratoria gestuurd. De laboratoria analyseerden de RMs volgens een gedetailleerd protocol in een periode van 3 maanden. Alle resultaten werden naar de partners gestuurd, gecontroleerd op compleetheid en statistisch geanalyseerd door drie statistici. De technische data werden gecontroleerd en samengevat door een microbioloog. Alle resultaten werden besproken tijdens een plenaire vergadering met alle deelnemers, waar ook afspraken werden gemaakt over de definitieve statistische analyse op de vastgestelde data (‘technisch valide data’). De belangrijkste conclusie was dat certificering van de microbiologische RMs haalbaar was voor alle tot doel gestelde parameters op de gewenste besmettingsniveaus (voor de Drinkwaterrichtlijn en voor de Zwemwaterrichtlijn).
Een aantal aanbevelingen voor toekomstige certificeringsstudies van microbiologische RMs werden gedaan. Samengevat:

- Selecteer getrainde laboratoria, met een kwaliteitssysteem;
- Voorzie deelnemers van gedetailleerde instructies en vraag gedetailleerde informatie ten aanzien van technische aspecten;
- Gebruik gestandaardiseerde methoden (zoals ISO, EN);
- Houd de benedengrens van RMs voor kwantitatieve (telling) methoden ≥ 10 cfp;
- Test stabiliteit van de partijen RMs bij verschillende temperaturen;
- Test homogeniteit van de partijen RMs na productie binnen één laboratorium en tijdens de studie tussen laboratoria;
- Maak gedetailleerde en practische instructies voor de CRM gebruikers.

Summary

The European project ‘MICROCRM’ started on 1 February 2001 and lasted until 1 November 2003. The acronym MICROCRM stands for: ‘Microbiological Certified Reference Materials in support of EU water legislation, performance testing and laboratory quality control’. The aim of the MICROCRM project was to determine the conditions that are necessary to produce and certify key water microbiological reference materials (RMs) that will support EU Water legislation (Drinking-water and Bathing-water Directives).
Feasibility certification studies were carried out in 2002. For this purpose the three partners in the project each produced batches of one of three different types of microbiological reference materials (RMs): capsules, lenticules, pastilles. Of each type of RM, eight batches, containing different bacterial strains, were prepared. All batches of RMs were checked for mean contamination level, homogeneity and stability. A set number of all types and batches of RMs were sent to thirteen (pre-selected and trained) European laboratories. The laboratories performed the analyses of the RMs according to detailed protocols in a period of 3 months. All results were sent to the partners, checked for completeness and statistically analysed by three statisticians. The technical data were checked and summarised by a microbiologist. All results were discussed at a plenary meeting with the participants, where agreements were made for final statistical analyses on agreed data (‘technical valid data’). The main conclusion was that certification of the microbiological RMs was feasible for all target parameters at the desired concentration levels (for the Drinking-water Directive and the Bathing-water Directive).
Several recommendations for future certification studies of microbiological RMs were made. Summarised:

- Select trained laboratories, with a quality system;
- Provide participants with detailed instructions and ask detailed information concerning technical aspects;
- Use well established standard methods (like ISO, EN);
- Keep lower limit of RMs for quantitative (enumeration) methods ≥ 10 cfp;
- Test stability of the batches of RMs at different temperatures;
- Test homogeneity of the batches of RMs after production within one laboratory and during the study between laboratories;
- Prepare detailed and practical instructions for CRM users.

1. Introduction

The European project 'MICROCRM' started on 1 February 2001 and lasted until 1 November 2003. The acronym MICROCRM stands for: 'Microbiological Certified Reference Materials in support of EU water legislation, performance testing and laboratory quality control'. The aim of the MICROCRM project was to determine the conditions that are necessary to produce and certify key water microbiological reference materials (RMs) that will support EU Water legislation (Drinking-water and Bathing-water Directives).
The workplan of the project followed several steps:

1. Description of objective specifications for microbiological CRMs fit for purpose (in support of EU water legislations);
2. Research, production and testing phase of key batches of microbiological RMs;
3. Training and harmonisation session between participant laboratories in a central laboratory facility;
4. Certification feasibility studies.

Ad 1) is described in Mooijman *et al*., 2001.
Ad 2) is described in separate reports for the three different types of reference materials. The three partners in the project each produced batches of one of three different types of microbiological reference materials (RMs):

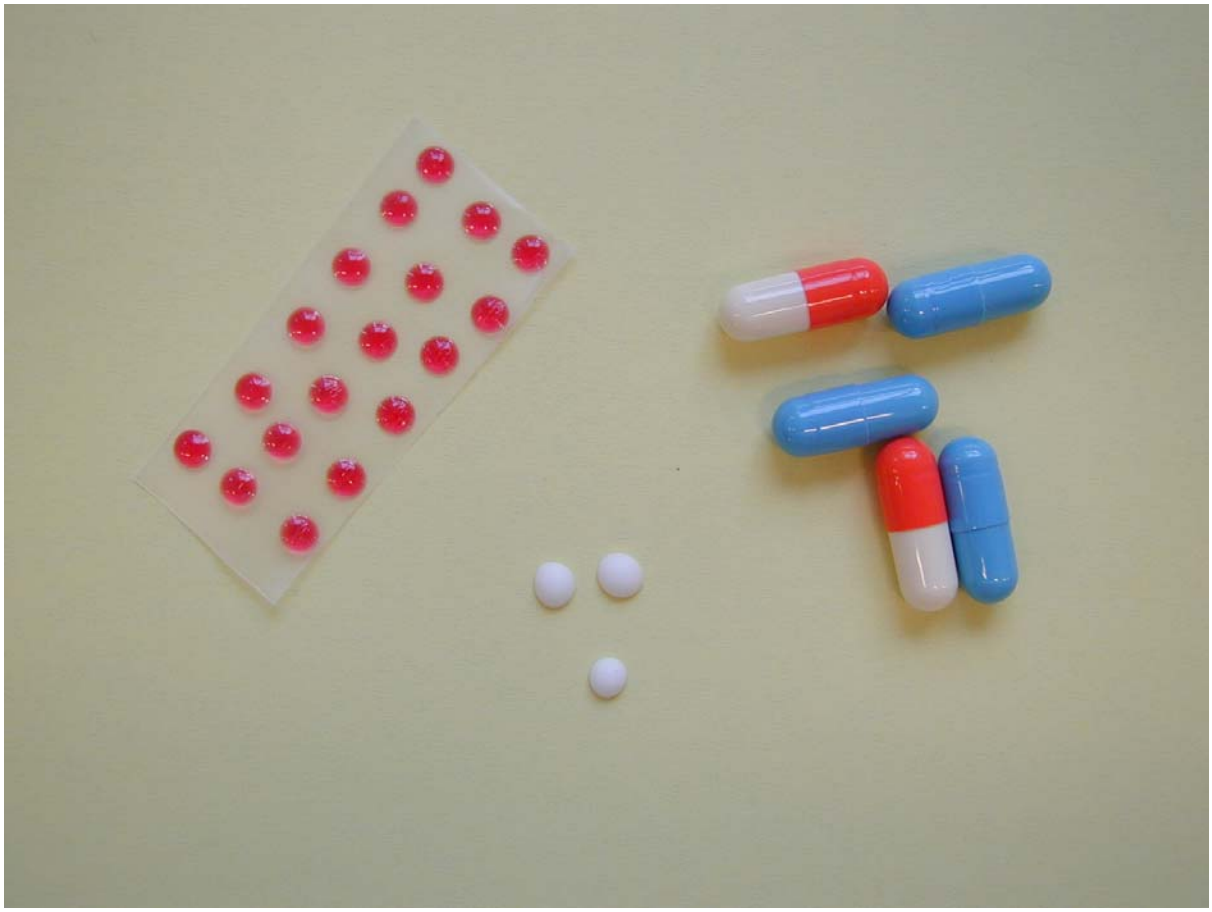
- National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands; Microbiological Laboratory for Health Protection (MGB), produced capsules (Mooijman *et al*., 2003);
- Public Health Laboratory Service (PHLS) Board, Newcastle, United Kingdom, produced lenticules (Tharagonnet *et al.*, 2004);
- Institut Pasteur de Lille (IPL), France; Water & Environment Department, produced pastilles (Pierzo *et al*., 2004).

Ad 3) is described in Simonart *et al*., 2003.
Ad 4) is described in this report.

2. Participants

2.1 Preparation and control of reference materials

- National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands; Microbiological Laboratory for Health Protection (MGB), produced capsules;
- Public Health Laboratory Service (PHLS) Board, Newcastle, United Kingdom, produced lenticules;
- Institut Pasteur de Lille (IPL), France; Water & Environment Department, produced pastilles.



Picture 1 *Three types of reference materials: lenticules (left), pastilles (middle) and capsules (right)*

2.2 Participants feasibility studies

- Hygiene Institute University of Vienna	Vienna	AT
- Christian Albrecht University of Kiel, Institut für Hygiene und Umweltmedizin	Kiel	DE
- Direccion de Salud Publica, Departemento de Sanida Gobierno Vasco, Laboratorio Normativo de Salud Publica	Bilbao	ES
- City of Helsinki, Environment Centre, Environmental Laboratory	Helsinki	FI
- Institut Pasteur de Lille, Water and Environment Department	Lille	FR
- Public Health Laboratory Service, Newcastle	Newcastle	GB
- National School of Public Health	Athens	GR
- National Institute for Hygiene, Department of Water Hygiene	Budapest	HU
- East Coast Area Health Board (ECAHB), Public Analyst Laboratory, Sir Patrick Dun's	Dublin	IE
- Instituto Superiore di Sanita Governative	Rome	IT
- National Institute for Public Health and the Environment (RIVM), Microbiological Laboratory for Health Protection	Bilthoven	NL
- Instituto Nacional de Saude Dr Ricardo Jorge, Laboratorio de Microbiologia de Aguas	Lisboa	PT
- Laborex 2000 SRL, Central Laboratory for Tests and Analysis, Microbiological Laboratory	Bucharest	RO

3. Materials and Methods

3.1 Reference materials

The three partners in the project each produced batches of one of three different types of microbiological reference materials (RMs); see 2.1. In Table 1 an overview is given on the selected micro-organisms which were included in the different types of RMs. It is also indicated with which analytical method the RMs were analysed, the target level in the final analytical portions and which water directive the RM would support. Each partner prepared its own type of RM with the selected strains and at the selected target level. Each batch of reference materials was checked for homogeneity and for stability according to the procedures as described in Mooijman *et al*., 2001. The results of all batches are described in three separate reports, one per type of RM (see Chapter 1).
During the production and control of the batch of capsules containing *Pseudomonas aeruginosa* it was already noted that the batch was not sufficiently stable for use during the feasibility certification studies. It was therefore decided on forehand not to use this batch of RMs (also see Mooijman *et al*., 2003).

Table 1 *Selected micro-organisms and target levels in the final analytical portions of the reference materials (RMs) and the methods for analysing the RMs*

EU Water Directive[1]	Micro-organism	Analytical method	Target concentration cfp/volume[2]
BW	*Escherichia coli*	ISO 9308-3	400 /100ml
BW	Intestinal *Enterococci*	ISO 7899-1	200 /100ml
DW	*Clostridium perfringens*	ISO(WD) 6461-2 without heating	50 / 100ml
DW	Culturable organisms (22°C)	ISO 6222	50 / 1ml
DW	Culturable organisms (36°C)	ISO 6222	50 / 1ml
DW	*Escherichia coli*	ISO 9308-1 [3]	50 / 100ml
DW	Intestinal *Enterococci*	ISO 7899-2	50 / 100ml
DW	*Pseudomonas aeruginosa*	(pr)EN 12780	50 / 50ml (pastilles) 50 / 100 ml (lenticules)

[1]: BW: Bathing Water. Information based on 'Communication from the Commission to the European Parliament and the Council, Developing a New Bathing Water Policy', Brussels 21.12.2000; DW: Drinking Water (Council Directive 93/83/EC of 3 November 1998 on the quality of water intended for human consumption);
[2]: cfp: colony forming particles; [3]: Only the standard test on Lactose TTC agar

3.2 Outline of the collaborative studies

One to two months before the feasibility certification studies, the 13 participating laboratories were trained in the use of the three types of RMs with the relevant methods at the central laboratory of Institut Pasteur de Lille. This made the laboratories more acquainted with the materials and methods. For more details of the training study, see Simonart *et al*., 2003.
Each participating laboratory received in March 2002 a final set of instructions and parcels of the three partners (see 3.1), containing the different reference materials. Each parcel also contained a small electronic temperature recorder, which has recorded the temperature during mailing. The recorders had to be returned to Institut Pasteur de Lille for reading.
In the period 1 April 2002 – 30 June 2002, each laboratory analysed the RMs according to the instructions. The results of each type of RM had to be reported in Excel sheets and returned (by e-mail) to the relevant partner after finishing the analyses. Each partner checked the results for completeness and sent them to the relevant statistician. Each statistician performed statistical analyses, which were combined in a central meeting of the statisticians. Results of all participants were presented and discussed in a central meeting with the participants (February 2003). The conclusions from this meeting were used for the final analyses.

To perform the collaborative studies, the participating laboratories received a large set of instructions, consisting of 11 (paper) documents and 3 Excel files:

- Protocol - Feasibility certification studies of microbiological reference materials (15-03-2002), Annex 1;
- SOP BCR-Water/001 (08-03-2002) Reconstitution of microbiological reference materials, consisting of gelatine capsules, in 10 ml solution. RIVM, Annex 2;
- RIVM/MGB-I001 (14-03-2002) Instructions for analysing microbiological reference materials, consisting of gelatine capsules, with different methods. RIVM, Annex 3;
- Lenticules-I002 (08-03-2002) Rehydration and preparation of lenticule discs before use. PHLS, Annex 4;
- SOP IPL/002 (15-03-2002) Rehydration and preparation of pastilles for use. IPL, Annex 5;
- Pastilles-I003 (15-03-2002) Instructions for analysing microbiological reference materials, consisting of pastilles, with different methods. IPL, Annex 6;
- SOP IPL/003 (15-03-2002) Instructions for quality control of media. IPL, Annex 7 ;
- Reporting form of technical data of the feasibility certification studies of microbiological reference materials (14-03-2002), Annex 8;
- Reporting form of count results of capsules of the feasibility certification studies of microbiological reference materials (14-03-2002), Annex 9;
- Reporting form of count results of lenticules of the feasibility certification studies of microbiological reference materials (14-03-2002), Annex 10;
- Reporting form of count results of pastilles of the feasibility certification studies of microbiological reference materials (draft 15-03-2002), Annex 11.

Excel files:
- Excel sheet for reporting data of the lenticules to PHLS/University of Newcastle;
- Excel sheet for reporting data of the pastilles to IPL;
- Excel sheet for reporting data of the capsules to RIVM-MGB.

The number of reference materials (RMs), which had to be analysed by the participating laboratories, was dependent on the method and the type of RM. An overview is given in Table 2.

Table 2 Number of 'Units' of each reference material (RM) to be analysed in the certification feasibility studies

RM Type	100 ml samples	1 ml samples
Lenticules	10 in singular	5 in duplicate
Pastilles	10 in singular	5 in duplicate
Capsules	5 in duplicate	5 in duplicate

Analysing an RM in duplicate meant that out of the RM-solution two sub-samples were taken and analysed separately.

Participating laboratories were free to plan the analyses in their own way, as long as it was performed in the period indicated (1 April – 30 June 2003). The analyses of one type of RM with its relevant method had to be performed on one day and not spreaded out over different days. It was permitted to analyse more than one type of RM and/or more than one method on one day. More details on the outline of the studies can be found in Annex 1.

3.3 Analyses of results

3.3.1 Screening of (technical) results

The participating laboratories had to record their data per type of RM on the relevant reporting forms. Furthermore they entered the data in the relevant Excel sheets. The reporting forms had to be sent by normal mail or by fax and the Excel files by e-mail to the relevant contact person (depending on the type of RM). The contact person checked the results for completeness and whether the data were correctly entered into the Excel sheets and sent the

results to the relevant statistician. The statisticians of the three partners agreed with each other on performing further analyses (see 3.3.2).
Each laboratory also sent a completed reporting form of technical data to one contact person. This contact person checked all technical data with the prescribed procedures.
At a central meeting with all participants (February 2003) all results were discussed. During this meeting first the technical observations were discussed before discussing the data. Where (large) deviations from the procedure criteria were observed (e.g. incubation time, incubation temperature, medium, etc,), the participants discussed the possible effects on the results. Any doubtful results were marked or discarded.

3.3.2 Statistical analyses of count results

Box and whisker plots
Of all data box and whisker plots were prepared. The results were presented per type of RM and per method. By this way of presentation, deviations in results (e.g. large variations, unexpected results in a laboratory), can easily be observed. The box plot results were discussed in combination with the technical data. Deviating results that could be explained by technical deviations were excluded for further analyses.

Certified values
In order to determine 'certified values' of an RM, all data were used from the certification study that were judged acceptable.
By 'certified values' is denoted, the range (interval) of values such that a laboratory working according to the same standards as the 13 participating trial laboratories should find a result that with 95% probability is contained within the interval of 'certified values'. Where appropriate, a distinction was made between a value consisting of a single count and a value consisting of the mean of 2 duplicate counts. The range for the latter kind of value would be narrower than that for a single count.

In order to estimate this range, it is noted that the results are influenced by several sources of variation:
1. Variation among (heterogeneity due to) laboratories. Some laboratories may systematically have higher or lower counts than the average, e.g. due to variations in transport conditions, or due to variations in quality of media or machinery;
2. Variation among units of one batch of RMs. This variation is always present, if only due to the discrete character of the organisms it contains. Poisson distribution is the lower bound of variation among units of one batch of RMs. However, due to e.g. properties of the manufacturing process, variation may exceed Poisson variation;

3. Variation between duplicate counts from units of one batch of RMs. Again, Poisson variation constitutes the lower bound of this variation, and again various circumstances may give rise to additional extra-Poisson heterogeneity or overdispersion.

In order to analyse the data a y = log(x+1) transformation was used. Calculated certification limits for this transformed variable followed by back transformation of certification limits to the normal scale.

For each y_{ijk} observation (that is the $\log(x_{ijk}+1)$ transform of the count x_{ijk} in the i-th lab, the j-th RM, and k-th count) its variance is written as:

$$\sigma_{ijk}^2 = \sigma^2(y_{ijk}) = \sigma_i^2 + \sigma_j^2 + \sigma_k^2$$

As the data are clearly nested (e.g. an RM is used in only one laboratory) PROC NESTED (SAS 8.2) was used to estimate the three variance components. For calculating the variance of the mean of duplicate counts the following formula was used:

$$\sigma_{ij.}^2 = \sigma^2(y_{ij.}) = \sigma_i^2 + \sigma_j^2 + \sigma_k^2/2$$

Certification limits were obtained by taking the estimated overall mean ± 2 σ_{ijk}, or (for means of duplicates), as mean ± 2 $\sigma_{ij.}$

Homogeneity
In the analysis described above, sources of variation are treated empirically, and no values for the variance components are of specific interest. However, these certification data can also be used to explore homogeneity of the RMs. For this the T_1 /df and T_2 /df statistics per laboratory can be used (formulas of T_1 and T_2 are given in Mooijman *et al*., 2003). On average, these values should be approximately 1 if relevant sources of variation do not exceed Poisson variation.
A complication arises with the microtiter plates. Results from these plates are expressed as MPN (Most Probable Number) which are not Poisson distributed.

These MPN can be analysed by considering that for each well the probability that it is positive equals:

$$\Pr(\text{well is positive}) = 1 - \Pr(0 \text{ organisms present}) = 1 - \exp(-q\lambda)$$

Where q denotes the quantity of solution used and λ denotes the concentration in the solution.

Hence,

$$\log(-\log(1-p)) = \log(q) + \log(\lambda)$$

and consequently these data can be analysed using a generalised linear model (GLM), treating log(q) as an offset and write either:

$$\log(\lambda_{ijk}) = a + lab_i + lab_i*RM_j$$

or:

$$\log(\lambda_{ijk}) = a + lab_i + lab_i*RM_j + lab_i*RM_j*replic_k$$

to detect either heterogeneities among RMs or between replicate measurements.

For this analysis, SAS PROC LOGISTIC was used.
Heterogeneities among laboratories were analysed by testing whether an interaction term between laboratory and RM was statistically significant.

In addition, homogeneity can be approached purely empirically, by estimating the T-value. This T-value is the limit below which the ratio (max/min) of two random results should be with 95% probability. Again, different situations can be considered, depending on whether results are from different laboratories or from the same one, and whether "results" are single results or the average of 2 duplicates. This value is easily estimated from the estimated variance components, by considering that:

$$var(y_1 - y_2) = 2var(y)$$

Thus on a logarithmic scale, the difference (i.e. the ratio on the untransformed, original, scale) between two values is with 95% probability less than (approximately) 2.8*standard deviation of (y). This standard deviation depends on the variance components involved, which in turn depends on whether two RMs are tested within the same laboratory or in different ones.

4. Results

4.1 Technical results

All technical data were checked with the prescribed procedures. Deviations were marked and further discussed with the participants. In case it was expected that deviations could have influenced the results, it was decided not to use these results for further analyses. The tables with technical results are given in Annex 12.

4.2 Data and statistics

4.2.1 Discussion of data

Most of the data are summarised in box and whisker plots and are given in Annex 13. Data that were obtained under largely deviating technical circumstances are not shown in the plots. In case the deviations in the technical circumstances were small or in case the results of a laboratory were deviating, but could not immediately be explained, the data are still shown in the plots bur are marked black. A summary of the discussion on these latter data is given below, together with the final decisions on which data were deleted for further analyses. Furthermore it was found for all methods that no relation between results and media manufacturers or batches and filter manufacturers or batches could be detected.

ISO 6222, Culturable organisms, cultured at 22 °C and at 36 °C (Anonymous, 1999a)
Laboratory 6: All results found with capsules incubated at both temperatures were 10 times lower than expected. The reason could not be traced anymore, but the explanation was thought in a pipetting error. It was decided to delete the data for further analyses.
Laboratory 8: The results found with pastilles, when incubated at 36 °C, were high and variable. A technical deviation here was the fact that during incubation stacks of 10 plates were made (while the advice was at maximum 6 plates). In case of high stacks of plates, uneven temperature distribution could occur in the plates, which might have caused the relatively high and variable count results. It was decided to delete the data for further analyses.

Table 3 Deleted data because of technical problems for ISO 6222, Culturable organisms incubated at 22 °C and at 36 °C

Lab	Deleted data	Reasons
6	Capsules, 22 °C and 36 °C (all data)	All results were 10 times lower than expected. Might have been a pipetting error (although not reported)
7	Lenticules and pastilles, 22 °C and 36 °C (all data)	No duplicate counts were made
8	Pastilles 36 °C (all data)	Very high and variable results. Might have been caused by high stacks of Petri dishes

ISO/WD 6461-2, Clostridium perfringens (Anonymous, 2001)

Many laboratories reported non-typical (white/pale) colonies when analysing the RMs for *Clostridium perfringens*. Especially when analysing lenticules, many laboratories (8/13) found only non-typical colonies. In Annex 13, box and whisker plots of typical and non-typical (atypical) colonies are given as well as of only typical colonies. It was decided to delete the results of non-typical colonies for further analyses. For the lenticules this would result in the fact that the data of only 4 laboratories (data of one more laboratory was deleted because of wrong incubation temperature) could be used for further analyses (see Table 4). It was therefore decided not to use the results of the lenticules for *Clostridium perfringens*.

Table 4 Deleted data because of technical problems for ISO/WD 6461-2, Clostridium perfringens

Lab	Deleted data	Reasons
1, 2, 3, 4, 7, 10, 11, 13	Lenticules (all data)	Colonies were non-typical
2	Pastilles (all data)	Colonies were non-typical
13	Pastilles (few non-typical colonies)	Some colonies were non-typical
2, 10, 11, 13	Capsules (all data)	Most of the colonies were non-typical
9	Lenticules, pastilles and capsules (all data)	Incubation temperature was 36 °C instead of 44 °C
2	Lenticules (all data)	Incubation time was ca 48 h instead of 24 h
7, 13	Lenticules, pastilles and capsules (all data)	Incubation time was ca 48 h instead of 24 h

ISO 7899-1, Intestinal Enterococci miniaturised MPN (Anonymous, 1998a)
No data were deleted because of technical problems. It was noted, however, that the variation in results of the pastilles was very high, but no data were deleted on forehand.

ISO 7899-2, Intestinal Enterococci membrane filtration (Anonymous, 2000a)
No discussion was necessary on the 'deviating' results of this method. Deleted data are summarised in Table 5.

Table 5 Deleted data because of technical problems for ISO 7899-2, Intestinal Enterococci membrane filtration

Lab	Deleted data	Reasons
1	Capsules (all data)	No growth was found. Might have been caused by an unknown technical error. Lab preferred to enter the results as missing.
8	Lenticules, unit 1	Count was 6 cfp, whereas for the other units ca 60 cfp was counted. Most probable a typing error

ISO 9308-1, Escherichia coli and coliforms, membrane filtration (Anonymous, 2000b) and ISO 9308-3, Escherichia coli, miniaturised MPN (Anonymous, 1998b)
Laboratory 9: The transport time of the capsules had been *ca* 15 days and the temperature during transport was *ca* 15 °C. Stability test experiments at different storage temperatures had shown that the number of culturable *E. coli* in the capsules would decrease when stored at elevated temperatures (> +5 °C; see Mooijman *et al.,* 2003). It was therefore decided not to use the data of the *E. coli* capsules of laboratory 9.
Furthermore it was noted that also for these methods the variation in results of the pastilles was very high, but no data were deleted on forehand.
The complete list of deleted data for both methods are given in Tables 6 and 7.

Table 6 Deleted data because of technical problems for ISO 9308-1, Escherichia coli and coliforms, membrane filtration

Lab	Deleted data	Reasons
9	Capsules (all data)	During transport the capsules were ca 15 days at ca 15 °C. Considering the stability test result this will have affect the data.
9	Lenticules, pastilles and capsules (all data)	LSA was used instead of LTTC
11	Lenticules, unit 6	Result should be indicated as missing ('blurred' colonies, could not be counted)
13	Lenticules, pastilles and capsules (all data)	Incubation time was ca 44 h instead of ca 21 h

Table 7 Deleted data because of technical problems for ISO 9308-3, Escherichia coli, miniaturised MPN

Lab	Deleted data	Reasons
9	Capsules (all data)	During transport the capsules were ca 15 days at ca 15 °C. Considering the stability test result this will have affect the data.

prEN 12780, Pseudomonas aeruginosa, membrane filtration (Anonymous, 1999b)
The batch of capsules containing *Pseudomonas aeruginosa* was not sufficiently stable to be used in the feasibility certification studies (see Mooijman *et al*., 2003). Therefore no data were available for the capsules.
The results of laboratory 4 were low when compared to the results of the other laboratories (especially in case of pastilles). As the quality control results of the medium of this laboratory was at the lower limit, it was concluded that the low results were most probable caused by poor quality of the medium and therefore the data were deleted for further analyses.

Table 8 Deleted data because of technical problems for prEN 12780, Pseudomonas aeruginosa, membrane filtration

Lab	Deleted data	Reasons
4	Lenticules and pastilles (all data)	Low results, which might have been caused by poor quality of the medium. Results of QC of medium were at the minimum lower limit.

4.2.2 Homogeneity

The variation between results within laboratories were calculated with T_1 (variation within one unit of a RM), T_2 (variation between units of one batch of RM) and T (ratio of max/min results) (see 3.3.2). Where T was calculated for the results found within laboratories and between laboratories.
The tables with T_1 and T_2 results per laboratory and per method are given in Annex 14. A summary of the T_2 results per type of RM and per method is given in Figures 1-3. In these figures the values of T_2 divided by the number of degrees of freedom (df) are given. In case of a Poisson distribution $T_2/df = 1$. However, in daily practice the variation between units of one batch of RMs will be higher than a Poisson distribution and a value of $T_2/df = 2$-3 would still be well acceptable.

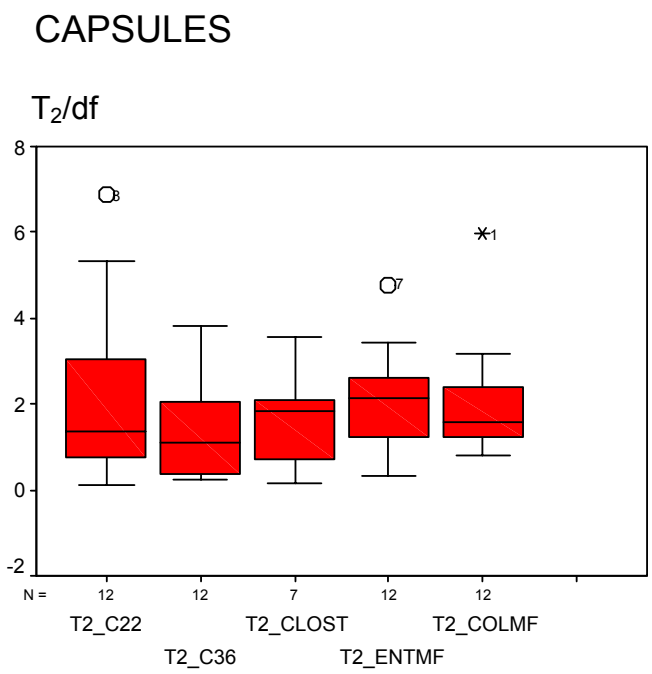


Figure 1 *Results of T_2/df of all laboratories (accepted data) for capsules analysed with ISO 6222, 22 °C (T2_C22), ISO 6222, 36 °C (T2_C36), ISO/WD 6461-2 (T2_CLOST), ISO 7899-2 (T2_ENTMF) and ISO 9308-1 (T2_COLMF)*

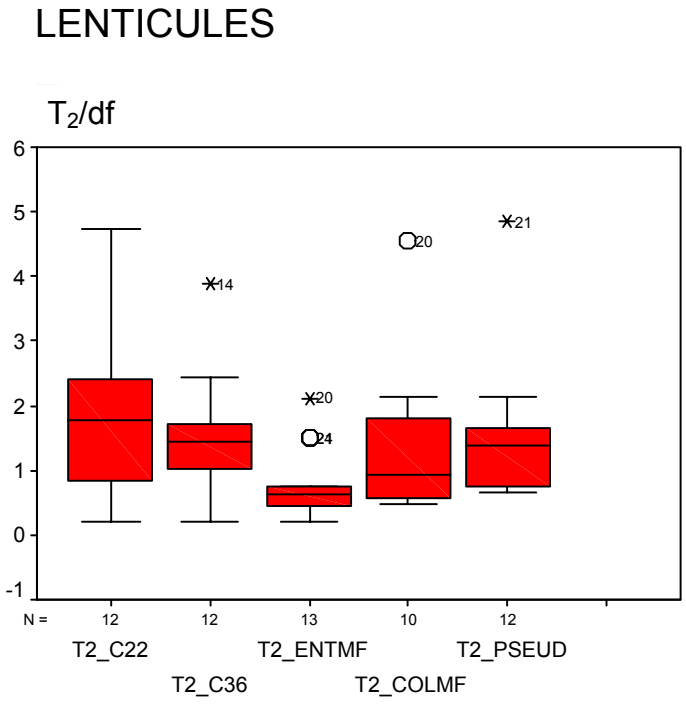


Figure 2 *Results of T_2/df of all laboratories (accepted data) for lenticules analysed with ISO 6222, 22 °C (T2_C22), ISO 6222, 36 °C (T2_C36), ISO 7899-2 (T2_ENTMF), ISO 9308-1 (T2_COLMF) and prEN 12780 (T2_PSEUD)*

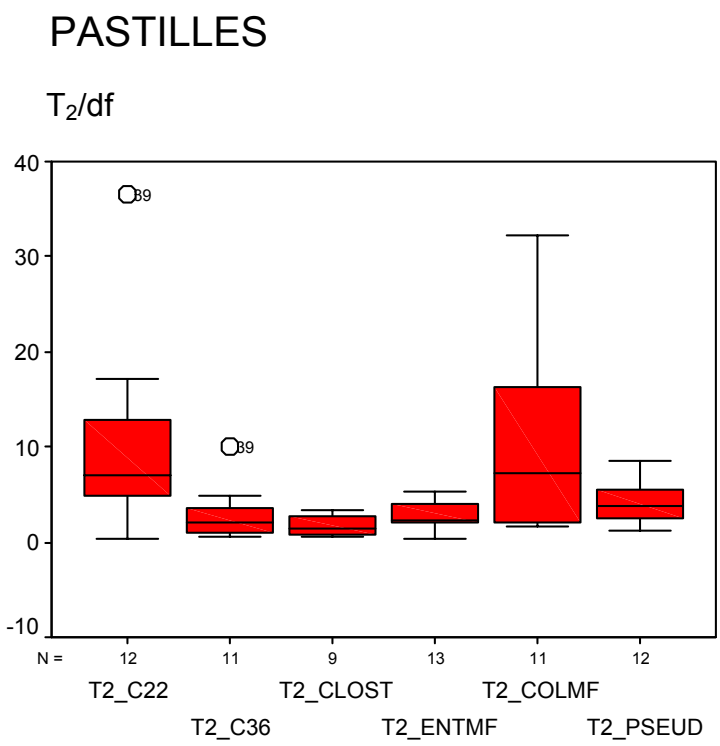


Figure 3 *Results of T_2/df of all laboratories (accepted data) for lenticules analysed with ISO 6222, 22 °C (T2_C22), ISO 6222, 36 °C (T2_C36), ISO/WD 6461-2 (T2_CLOST), ISO 7899-2 (T2_ENTMF), ISO 9308-1 (T2_COLMF) and prEN 12780 (T2_PSEUD)*

The T_1 and T_2 values of the miniaturised MPN methods were calculated in a slightly different way than for the other method (see 3.3.2). For the miniaturised MPN methods only T_1 and T_2 values for all laboratories were calculated (per type of RM and per method). The results are given in Table 9.

Table 9 *T_1 and T_2 values of the miniaturised MPN methods per type of RM and per method for all laboratories*

ISO-method	Capsules				Lenticules		Pastilles	
	df (T_1)	T_1/df	df (T_2)	T_2/df	df	T_2/df	df	T_2/df
7899-1 Intestinal *Enterococci*	65	0.81	52	0.79	117	1.21	117	2.95
9308-1 *E. coli*	60	1.06	48	1.03	117	1.32	113	9.41

df: degrees of freedom

In Table 10 the T-values (within laboratories and between laboratories) are given per type of RM and per method. The T-values aimed at were:

- within laboratories: ≤ 3 (preferable ≤ 2)
- between laboratories: ≤ 4 (preferable ≤3)

Table 10 T-values (ratio's of max/min results) within (T_{within}) and between ($T_{between}$) laboratories per type of RM and per method for all laboratories

method	Capsules		Lenticules		Pastilles	
	T_{within}	$T_{between}$	T_{within}	$T_{between}$	T_{within}	$T_{between}$
ISO 6222 cult. org. 22 °C	1.46	1.56	1.42	1.57	2.45	3.24
ISO 6222 cult. org. 36 °C	1.34	1.52	1.38	1.69	2.08	2.26
ISO/WD 6461-2 *Cl. perfringens*	1.36	1.73	na	na	3.09	4.02
ISO 7899-1 *Intestinal Enterococci* mpn	2.20	2.58	2.24	2.26	3.66	12.1
ISO 7899-2 *Intestinal Enterococci* mf	1.50	3.76	2.05	2.14	1.98	3.04
ISO 9308-1 *E. coli* mf	1.56	3.09	1.51	1.58	2.78	11.5
ISO 9308-3 *E. coli* mpn	1.61	2.07	1.92	2.15	7.99	38.2
pr EN 12780 *Ps. aeruginosa*	na	na	2.28	3.17	2.79	4.51

na: not applicable
cult. org. 22 °C (36 °C): culturable organisms, incubated at 22 °C (at 36 °C);
mpn: most probable number (miniaturised MPN method); mf: membrane filtration

4.3 Certified values of the feasibility studies

After exclusion of technically invalid results (4.2.1) the 'certified' values and its 95% confidence intervals were calculated per type of RM and per method. The results are given in Tables 11-13 and in Figures 4-9. The results of the miniaturised methods (ISO 7899-1 and 9308-1) are given in separate figures as the concentration levels differ from the other methods.

If the materials would have really been certified, a user of the CRM could analyse one unit of a CRM and check whether the result is within the limits with 95% confidence.

Table 11 *Certified values and 95% confidence intervals calculated from technically accepted data of **capsules***

Method	df labs	df capsules	df replicates	Certified cfp	95% c.i. (cfp) lower	upper
ISO 6222 cult. org. 22 °C	11	48	60	58.6	40.2	85.1
ISO 6222 cult. org. 36 °C	11	48	60	59.0	41.2	84.4
ISO/WD 6461-2 *Cl. perfringens*	6	28	35	64.9	42.0	100.0
ISO 7899-1 *Intestinal Enterococci* mpn	12	52	65	178.5	79.5	399.2
ISO 7899-2 *Intestinal Enterococci* mf	11	48	60	46.3	17.5	120.2
ISO 9308-1 *E. coli* mf	10	44	55	43.2	18.0	101.8
ISO 9308-3 *E. coli* mpn	11	48	60	563.8	312.8	1015.4
pr EN 12780 *Ps. aeruginosa*[1]	na	na	na	na	na	na

df: degrees of freedom;
cfp: colony forming particles;
c.i.: confidence interval;
na: not applicable;
cult. org. 22 °C (36 °C): culturable organisms, incubated at 22 °C (at 36 °C);
mpn: most probable number (miniaturised MPN method);
mf: membrane filtration;
[1]: Batch of RMs containing *Pseudomonas aeruginosa* was not stable and was therefore not used for feasibility certification study.

*Table 12 Certified values and 95% confidence intervals calculated from technically accepted data of **lenticules***

Method	df labs	df lenticules	df replicates	Certified cfp	95% c.i. (cfp) lower	upper
ISO 6222 cult. org. 22 °C	11	48	60	58.7	40.7	84.6
ISO 6222 cult. org. 36 °C	11	48	60	59.8	39.6	89.9
ISO/WD 6461-2 *Cl. perfringens*[1]	na	na	na	Na	Na	Na
ISO 7899-1 *Intestinal Enterococci* mpn	12	117	na	256.8	143.2	460.0
ISO 7899-2 *Intestinal Enterococci* mf	12	116	na	72.6	58.2	90.4
ISO 9308-1 *E. coli* mf	10	98	na	78.4	56.4	108.9
ISO 9308-3 *E. coli* mpn	12	117	na	461.2	266.8	797.0
pr EN 12780 *Ps. aeruginosa*	11	108	na	23.4	9.9	53.7

df: degrees of freedom;
cfp: colony forming particles;
c.i.: confidence interval;
na: not applicable;
cult. org. 22 °C (36 °C): culturable organisms, incubated at 22 °C (at 36 °C);
mpn: most probable number (miniaturised MPN method);
mf: membrane filtration;
[1]: Number of (technically) accepted data was too small (many atypical colonies reported) to calculate a certified value.

*Table 13 Certified values and 95% confidence intervals calculated from technically accepted data of **pastilles***

Method	df labs	df pastilles	df replicates	Certified cfp	95% c.i. (cfp) lower	upper
ISO 6222 cult. org. 22 °C	11	48	60	54.1	22.0	131.0
ISO 6222 cult. org. 36 °C	10	44	55	14.5	6.7	30.2
ISO/WD 6461-2 *Cl. perfringens*	8	81	na	9.1	2.7	26.2
ISO 7899-1 *Intestinal Enterococci* mpn	12	117	na	281.0	46.6[1]	1671.2[1]
ISO 7899-2 *Intestinal Enterococci* mf	12	117	na	56.6	25.7	112.8
ISO 9308-1 *E. coli* mf	10	99	na	52.7	8.4[1]	306.4[1]
ISO 9308-3 *E. coli* mpn	11	107	na	449.0	32.3[1]	6076.2[1]
pr EN 12780 *Ps. aeruginosa*	11	108	na	35.3	11.7	102.6

df: degrees of freedom;
cfp: colony forming particles;
c.i.: confidence interval;
na: not applicable;
cult. org. 22 °C (36 °C): culturable organisms, incubated at 22 °C (at 36 °C);
mpn: most probale number (miniaturised MPN method);
mf: membrane filtration;
[1]: (very) broad confidence interval

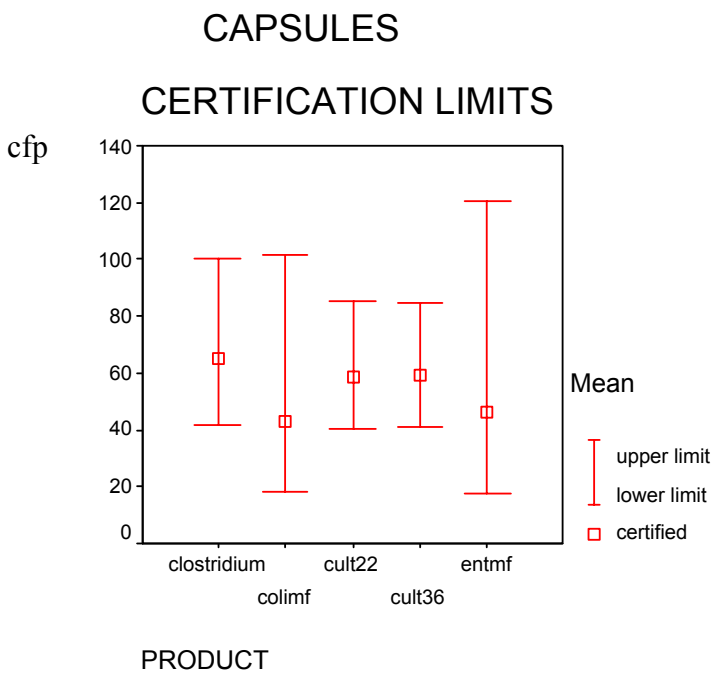


Figure 4 *Certified values and 95% confidence intervals calculated from technically accepted data of **capsules** with ISO 6222 (cult22 and cult36), ISO/WD 6461-2 (clostridium), ISO 7899-2 (entmf) and ISO 9308-1 (colimf)*

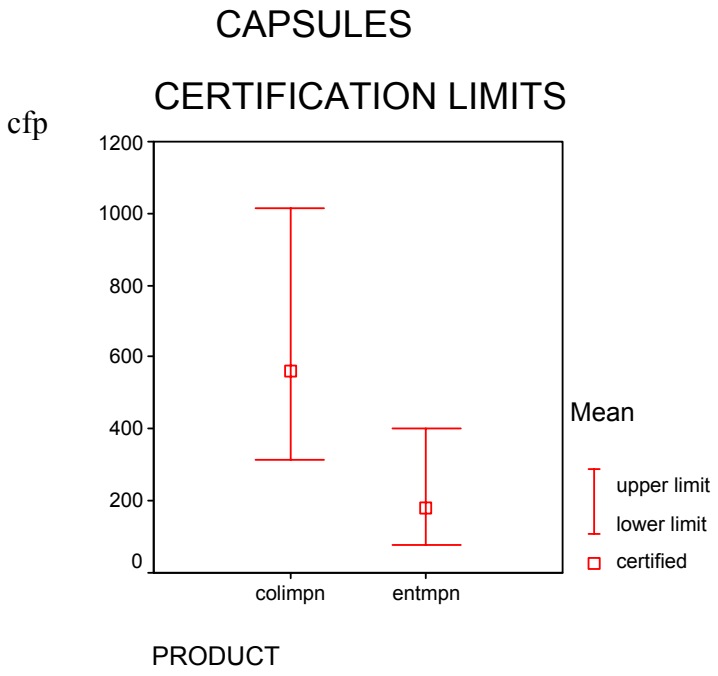


Figure 5 *Certified values and 95% confidence intervals calculated from technically accepted data of **capsules** with, ISO 7899-1 (entmpn) and ISO 9308-3 (colimpn)*

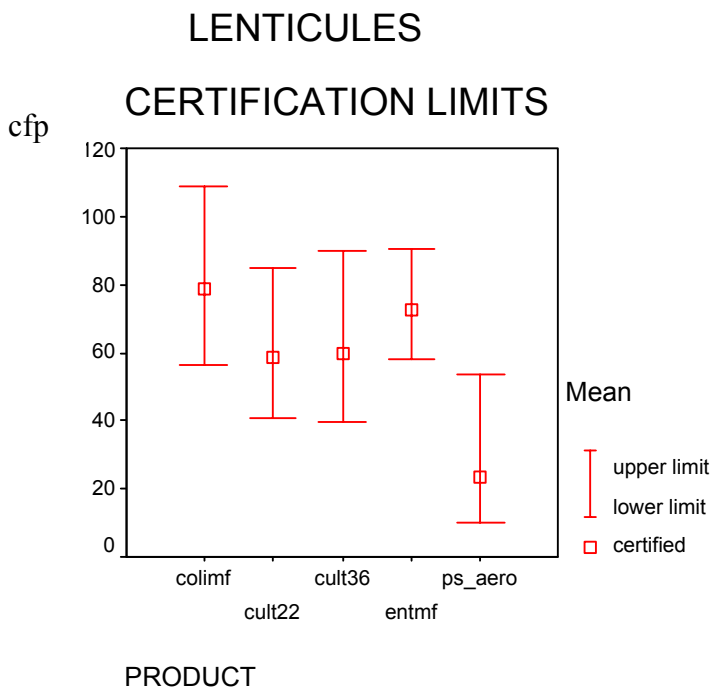


Figure 6 *Certified values and 95% confidence intervals calculated from technically accepted data of **lenticules** with ISO 6222 (cult22 and cult36), ISO 7899-2 (entmf),ISO 9308-1 (colimf) and prEN 12780 (ps_aero)*

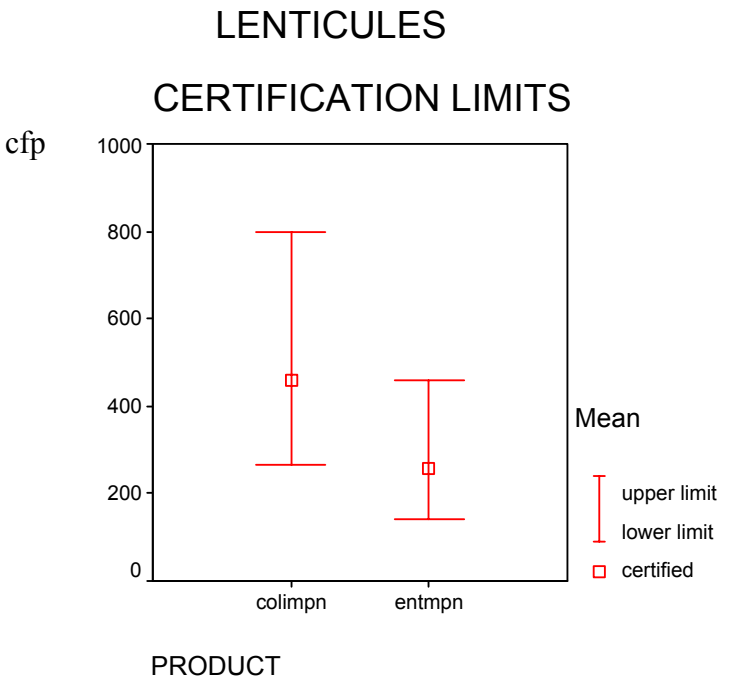


Figure 7 *Certified values and 95% confidence intervals calculated from technically accepted data of **lenticules** with, ISO 7899-1 (entmpn) and ISO 9308-3 (colimpn)*

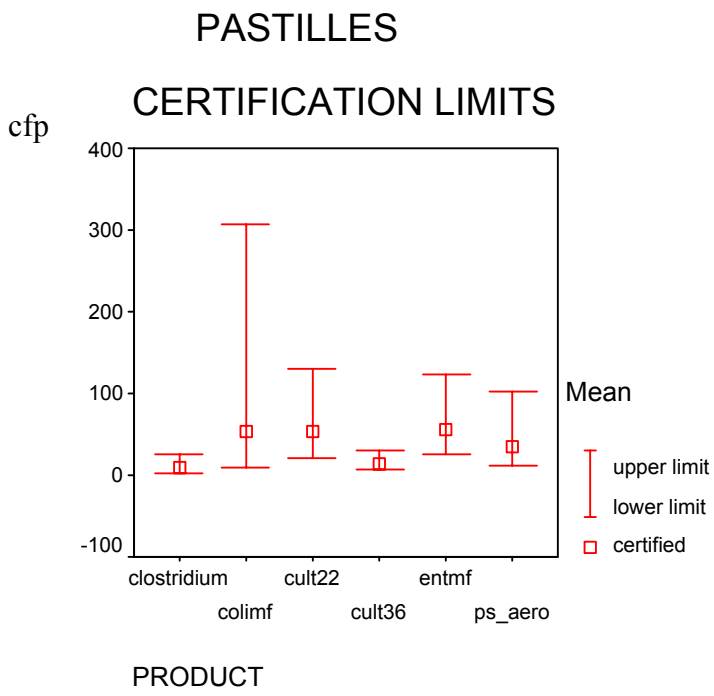


Figure 8 *Certified values and 95% confidence intervals calculated from technically accepted data of **pastilles** with ISO 6222 (cult22 and cult36), ISO/WD 6461-2 (clostridium), ISO 7899-2 (entmf) ISO 9308-1 (colimf) and prEN 12780 (ps_aero)*

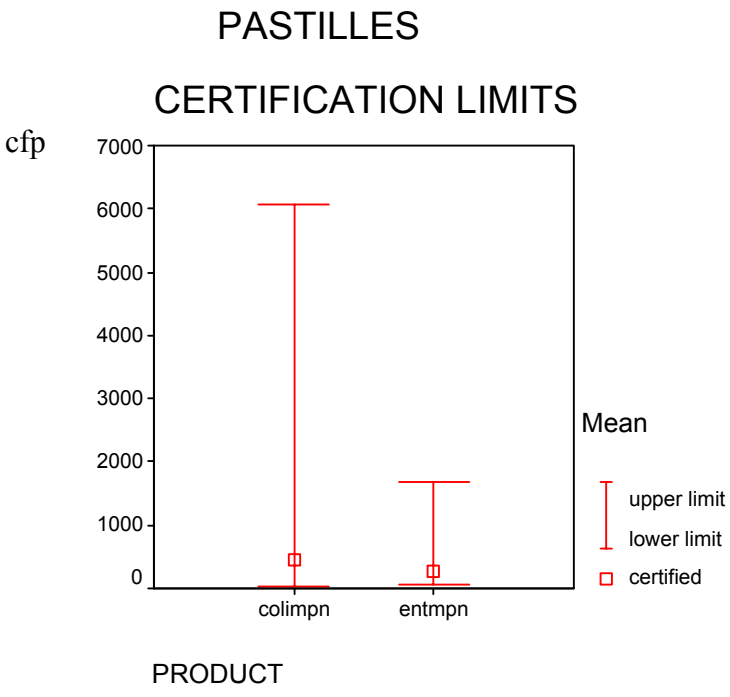


Figure 9 *Certified values and 95% confidence intervals calculated from technically accepted data of **pastilles** with, ISO 7899-1 (entmpn) and ISO 9308-3 (colimpn)*

5. Discussion and conclusions

Before the feasibility certification studies were performed, the participating laboratories had been trained with the three types of RMs and with the 8 different methods. This gave the laboratories the opportunity to become acquainted with the work. Technical problems, which existed during the feasibility studies, were in general not caused by problems with handling of the RMs or with applying the methods, but were more general technical problems which might also exist in daily practice.
Below, the discussion and conclusions per method are given.

ISO 6222, Culturable organims, cultured at 22 °C and at 36 °C (Anonymous, 1999a)
After exclusion of the technically invalid results (see Table 3), the results of all three type of RMs were sufficiently homogeneous to calculate certified values (see 4.3).

ISO/WD 6461-2, Clostridium perfringens (Anonymous, 2001)
The ISO procedure for the enumeration of *Clostridium perfringens* used during the feasibility studies was still a (working) draft document. The described procedure in this ISO was obviously not yet a very robust method. Especially the analyses of the lenticules showed many non-typical colonies, while the RMs did contain *Clostridium perfringens*. Also with the other type of RMs problems with non-typical colonies were reported. The problems detected during the study were summarised and it was discussed during the plenary meeting how the procedure might be optimised. All information related to this method was sent to the working group of the relevant ISO committee, dealing with ISO 6461-2. In this way the information of the study can be used to optimise the ISO procedure. However, this was not the aim of the feasibility certification study. The conclusion related to certification was that it would be difficult to produce certification data from such a non-robust method. Of the remaining (accepted) data some certified values were produced for the capsules and the pastilles (see 4.3), but not for the lenticules, as of this latter batch of RM too few data were left for further analyses.

ISO 7899-1, Intestinal Enterococci miniaturised MPN (Anonymous, 1998a)
For this method no data were excluded because of technical deviation. However, the variation in results between laboratories found with the batch of pastilles was too high to be certifiable (see 4.3). It was therefore concluded that this batch of pastilles could not be certified for ISO 7899-1.

ISO 7899-2, Intestinal Enterococci membrane filtration (Anonymous, 2000a)
After exclusion of the technically invalid results (see Table 5), the results of all three type of RMs were sufficiently homogeneous to calculate certified values (see 4.3).

ISO 9308-1, Escherichia coli and coliforms, membrane filtration (Anonymous, 2000b) and ISO 9308-3, Escherichia coli, miniaturised MPN (Anonymous, 1998b)
In stability studies of the three types of RMs containing *Escherichia coli,* it was shown that a decrease in the number of cfp would occur when the materials are stored at elevated temperatures (see Mooijman *et al*., 2003, Pierzo *et al*., 2004 and Tharagonnet *et al.*, 2004). Therefore it would be necessary to transport this type of RM at low temperatures (preferably < +5 °C) in a short period of time (preferably < 4 days).
After exclusion of the technically invalid results (see Tables 6 and 7), the results of the capsules and lenticules were sufficiently homogeneous to calculate certified values (see 4.3). However, for the batches of pastilles the results found with both methods were very variable (see 4.3). Therefore it was concluded that both batches of pastilles containing *Escherichia coli* could not be certified for ISO 9308-1 and ISO 9308-3.

prEN 12780, Pseudomonas aeruginosa, membrane filtration (Anonymous, 1999b)
The batch of capsules containing *Pseudomonas aeruginosa* was not used for the feasibility studies, as it was already noted at the control of the batch that the material was not sufficiently stable (see Mooijman *et al*., 2003).
After exclusion of the technically invalid results (see Table 8), the results of the lenticules and pastilles were sufficiently homogeneous to calculate certified values (see 4.3).

Summary conclusions

- Certification of microbiological RMs was shown to be feasible for all target parameters at the desired concentration levels (for the Drinking water Directive and the Bathing water Directive);
- Only a few batches of the three types of RMs were not certifiable (one batch of capsules out of seven, one batch of lenticules out of seven and three batches of pastilles out of eight);
- No major technical problems existed during the studies, except with ISO/WD 6461-2 (*Clostridium perfringens*);
- Stability of the (C)RMs is dependent on the strain, especially when stored at elevated temperatures;
- The batches of capsules and lenticules were during the present studies globally more robust than some batches of pastilles. It may be noted however, that the capsules and lenticules have a longer research history than the pastilles.

Recommendations for future certification studies of microbiological RMs

- Select trained laboratories (trained for use of RMs and analytical procedures), or train laboratories before performing the study, with a quality system (preferably accredited according to ISO 17025);
- Provide participants with detailed instructions and ask detailed information concerning technical aspects. The latter information will be necessary for discussion on acceptance of data;
- Use well established standard methods (like ISO, EN);
- Keep lower limit of RMs for quantitative (enumeration) methods ≥ 10 cfp (as the precision of results will become low at <10 cfp);
- Test stability of the batches of RMs at different temperatures (especially at elevated temperatures) to set criteria per type of RM for transport (concerning temperature and time);
- Test homogeneity of the batches of RMs after production within one laboratory and during the study between laboratories. Variation between units of one batch of RM will in general be larger than a Poisson distribution. Alternatively 'T' (see 3.3.2) can be used to test homogeneity. Criteria for the homogeneity can be as follows:
  - after production (one batch of RMs): T ≤ 3 (preferable ≤ 2) at α=0,05;
  - between laboratories (one batch of RMs): T≤ 4 (preferable ≤ 3) at α=0,05;
- Prepare detailed and practical instructions for CRM users.

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Anonymous. 1998a. ISO 7899-1 Water quality – Detection and enumeration of intestinal enterococci in surface and waste water – Part 1: Miniaturized method (Most Probable Number) by inoculation in liquid medium. International Organisation for Standardisation, Geneva, Switzerland.

Anonymous. 1998b. ISO 9308-3 Water quality – Detection and enumeration of *Escherichia coli* and coliform bacteria in surface and waste water – Part 3: Miniaturized method (Most Probable Number) by inoculation in liquid medium. International Organisation for Standardisation, Geneva, Switzerland.

Anonymous. 1999a. ISO 6222 Water quality – Enumeration of culturable micro-organisms – Colony count by inoculation in a nutrient agar culture medium. International Organisation for Standardisation, Geneva, Switzerland.

Anonymous. 1999b. prEN 12780 Water quality – Detection and enumeration of *Pseudomonas aeruginosa* by membrane filtration. European Committee for Standardization, Brussels, Belgium.

Anonymous. 2000a. ISO 7899-2 Water quality – Detection and enumeration of intestinal enterococci – Part 2: Membrane filtration method. International Organisation for Standardisation, Geneva, Switzerland.

Anonymous. 2000b. ISO 9308-1 Water quality – Detection and enumeration of *Escherichia coli* and coliform bacteria – Part 1: Membrane filtration method. International Organisation for Standardisation, Geneva, Switzerland.

Anonymous. 2001. ISO/WD 6461-2 Water quality – Detection and enumeration of *Clostridium perfringens* – Part 2: Method by membrane filtration. International Organisation for Standardisation, Geneva, Switzerland.

Mooijman KA, Stewardson D, Lightfoot N and Simonart T. MICROCRM. Report on WP1: Objectives specification, June 2001. National Institute for Public Health and the Environment, Bilthoven, the Netherlands; University of Newcastle, United Kingdom; Public Health Laboratory Service (PHLS) Board, Newcastle, United Kingdom ; Institut Pasteur de Lille, Water & Environment Dpt, Lille, France. Published as part of Progress report MICROCRM European Union Contract G6RD-CT-200-00264, August 2002, and as Annex 2 of Mooijman *et al*., 2003

Mooijman KA, During M and Nagelkerke NJD. MICROCRM: Preparation and control of batches of microbiological reference materials constisting of capsules. National Institute for Public Health and the Environment, Bilthoven, the Netherlands. RIVM report no 250935001, 2003.

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Tharagonnet D. MICROCRM: Preparation and control of batches of microbiological reference materials constisting of lenticules. European Union Contract G6RD-CT-200-00264, Public Health Laboratory Service (PHLS) Board, Newcastle, United Kingdom. 2004.

Mailing list

1 EU DG Research-Growth, Mrs. Dr. D. Ramaekers
2 Director General of RIVM
3 Depot Nederlandse Publikaties en Nederlandse Bibliografie
4 Director Sector VCV, Prof. Dr. Ir. D. Kromhout
5 Head Microbiological Laboratory for Health Protection, Dr. Ir. A. Mensink
6-18 Participating laboratories
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Annex 1 Protocol

15-03-2002

PROTOCOL

FEASIBILITY CERTIFICATION STUDIES OF MICROBIOLOGICAL REFERENCE MATERIALS

Please read all instructions and the reporting form carefully before starting the trial.
Use only the final instructions (<u>not</u> draft versions)!
Fill in the reporting form during the work and not afterwards.

INTRODUCTION

Different types of microbiological reference materials are prepared by the three partners in the EU-project MICROCRM:
- Public Health Laboratory Services Newcastle, UK (PHLS) prepares lenticules;
- Institut Pasteur de Lille, Fr (IPL) prepares pastilles and
- The Microbiological Laboratory for Health Protection (MGB) of the National Institute of Public Health and the Environment (RIVM), Bilthoven, NL, prepares capsules.

The feasibility certification studies consider reference materials (RMs) which would support EU water legislations. In Table A.1.1 an overview is given on the selected micro-organisms which will be included in the different RMs. It is also indicated with which analytical method the RMs should be analysed, the target level in the final analytical portions and which water directive the RM would support. Each partner has prepared its own type of RM with the selected strains and at the selected target level.

The next protocol describes (together with a Standard Operation Procedure and Instructions for use) the procedures for the feasibility certification studies of the different RMs.

Table A.1.1 Selected micro-organisms and target levels in the final analytical portions of the reference materials (RMs) and the methods for analysing the RMs

EU Water Directive[1]	Micro-organism	Analytical method	Target concentration cfp/volume[2]
BW	*Escherichia coli*	ISO 9308-3	400 /100ml
BW	Intestinal enterococci	ISO 7899-1	200 /100ml
DW	*Clostridium perfringens*	ISO(WD) 6461-2 without heating	50 / 100ml
DW	Culturable organisms (22°C)	ISO 6222	50 / 1ml
DW	Culturable organisms (36°C)	ISO 6222	50 / 1ml
DW	*Escherichia coli*	ISO 9308-1 [3]	50 / 100ml
DW	Intestinal enterococci	ISO 7899-2	50 / 100ml
DW	*Pseudomonas aeruginosa*	(pr)EN 12780	50 / 100ml

[1]: BW: Bathing Water. Information based on 'Communication from the Commission to the European Parliament and the Council, Developing a New Bathing Water Policy', Brussels 21.12.2000; DW: Drinking Water (Council Directive 93/83/EC of 3 November 1998 on the quality of water intended for human consumption);
[2]: cfp: colony forming particles; [3]: Only the standard test on Lactose TTC agar

OUTLINE OF THE STUDY

Each participating laboratory will receive in March 2002 a final set of instructions and parcels of the three partners, containing the different reference materials. Each parcel will also contain a small electronic temperature recorder, which has recorded the temperature during mailing. The recorders need to be returned to Institute Pasteur in Lille for reading.
In the period 1 April 2002 – 30 June 2002, each laboratory will analyse the RMs according to the instructions. The results of each type of RM are reported in Excel sheets and returned (by e-mail) to the relevant partner after finishing the analyses. Each partner will statistically analyse the results of its own RMs. Results of all partners will be summarised in one (draft) report and discussed in a meeting with the participants. The conclusions from this meeting are used for the preparation of the final report. The RMs will not officially be certified, but all steps will be taken until certification. The results of the studies will be used to advise future certification studies of this type of RMs.

LIST OF INSTUCTIONS

Find below a (check)list of protocols, SOPs, instructions, Excel files etc., which each laboratory should have received to perform the feasibility certification studies. Each laboratory should have received 11 documents (on paper) by normal mail and 3 Excel files by e-mail.

Documents:

- Protocol - Feasibility certification studies of microbiological reference materials (15-03-2002);
- SOP BCR-Water/001 (08-03-2002) Reconstitution of microbiological reference materials, consisting of gelatin capsules, in 10 ml solution. RIVM;
- RIVM/MGB-I001 (14-03-2002) Instructions for analysing microbiological reference materials, consisting of gelatin capsules, with different methods. RIVM;
- Lenticules-I002 (08-03-2002) Rehydration and preparation of lenticule discs before use. PHLS;
- SOP IPL/002 (15-03-2002) Rehydration and preparation of pastilles for use. IPL;
- Pastilles-I003 (15-03-2002) Instructions for analysing microbiological reference materials, consisting of pastilles, with different methods. IPL;
- SOP IPL/003 (15-03-2002) Instructions for quality control of media. IPL ;
- Reporting form of technical data of the feasibility certification studies of microbiological reference materials (14-03-2002);
- Reporting form of count results of capsules of the feasibility certification studies of microbiological reference materials (14-03-2002);
- Reporting form of count results of lenticules of the feasibility certification studies of microbiological reference materials (14-03-2002);
- Reporting form of count results of pastilles of the feasibility certification studies of microbiological reference materials (draft 15-03-2002).

Excel files:

- Excel sheet for reporting data of the lenticules to PHLS/University of Newcastle;
- Excel sheet for reporting data of the pastilles to IPL;
- Excel sheet for reporting data of the capsules to RIVM-MGB.

CHRONOLOGICAL DESCRIPTION OF THE CERTIFICATION TRIAL

Date (2002)

March	Final instructions are sent to the participants (18/19 March). Each partner will send sufficient reference materials to each participant. Each parcel will also contain a small electronic temperature recorder (25/26 March). After arrival of the parcels: Inspect the parcels and note the date of arrival on the reporting form. Each temperature recorder should have been labelled with type RMs (e.g. 'capsules') and labcode (e.g. 'lab 9'). If not, please label each recorder with the relevant information. **Store the reference materials at (-20 ± 5) °C immediately after receipt**. Note date and time of storage on the reporting form. Acknowledge the receipt of each parcel to the relevant partner: - Lenticules to Danka Tharagonnet (PHLS-Newcastle): newdthar@north.phls.nhs.uk; - Pastilles to Tristan Simonart (IPL-Lille): tristan.simonart@pasteur-lille.fr; - Capsules to Kirsten Mooijman (RIVM/MGB-Bilthoven): kirsten.mooijman@rivm.nl. Return the three temperature recorders as soon as possible to: Tristan Simonart; Institute Pasteur Lille; Water and Environment department; 1, rue du Professeur Calmette; P.O.Box 245; 59019 Lille; France. In case of problems with a parcel also contact the above mentioned persons.

March-onwards	Control the temperature of the freezer every (working) day. If continuous temperature reading has been performed, please send a printout of the relevant period together with the reporting form on technical data. Else, give a list of min/max temperatures. Prepare glassware, reagents and isolation media of the analytical methods as described in Table A.1.1. Control, using the special RMs, the media to be used during the studies. Follow the enclosed instructions (SOP IPL/003, 15-03-2002). Quality control of microtiter plates is included in the process of the manufacturer and therefore does not need to be performed by the laboratory. Use own methods for the quality control of the membrane filters. **Use only media and filters that have been proven to be of good quality.**
1 April – 30 June	Perform the feasibility certification studies. Each laboratory can make its own scheme for analysing the RMs as long as all analyses are performed in the period **1 April – 30 June**. **Mind that the analyses of one type of RM with its relevant method should be performed on one day and not spreaded out over different days.** *Note the exact date of analyses for each method and each reference material on the reporting form of technical data.* *Note the count results in the relevant reporting form of count results and in the relevant Excel sheet.*
Before 5 July	Completed Excel sheets are e-mailed to the persons indicated in the relevant reporting forms, by the participating laboratories. Completed reporting form of technical data is sent by e-mail, fax or by normal mail to Kirsten Mooijman: kirsten.mooijman@rivm.nl; (fax) +31 30 274 4434; RIVM/MGB (Pb63); P.O.Box 1; 3720 BA Bilthoven, the Netherlands.
July - September	Statistical analysis of the results; Preparation of the draft reports.
Oct/Nov	Discussion of the results with participants.
Dec/Jan (2003)	Final statistical analysis; Preparation of final reports.

DETAILED DESCRIPTION OF THE TRIAL

General for volumes and weights: Unless otherwise stated, the accepted range of any measured value in this protocol is: stated value ± 5 %.

The number of reference materials (RMs) to be analysed depends on the method and the type of RM. An overview is given in Table A.1.2.

Table A.1.2 Number of 'Units' of each reference material (RM) to be analysed in the certification feasibility studies

RM Type	100 ml samples	1 ml samples
Lenticules	10 in singular	5 in duplicate
Pastilles	10 in singular	5 in duplicate
Capsules	5 in duplicate	5 in duplicate

Analysing an RM in duplicate means that out of the RM-solution two sub-samples are taken and analysed separately.

For the use of the capsules, SOP BCR-Water/001 (08-03-2002) and the instructions in RIVM/MGB-I001 (14-03-2002) should be followed.

For the use of the lenticules, Lenticules-I002 (08-03-2002) should be followed.

For the use of the pastilles, SOP IPL/002 (15-03-2002) and the instructions in Pastilles-I003 (15-03-2002) should be followed.

All analyses should be performed in the period **1 April – 30 June 2002**. Participating laboratories are free to plan the analyses in their own way, as long as it is performed in the period indicated. Mind that the analyses of one type of RM with its relevant method should be performed on one day and not spreaded out over different days. It is permitted to analyse more than one type of RM and/or more than one method on one day. Label all plates carefully and note all relevant information on the reporting form of technical data.

Before reading, the plates should be mixed in a random order and (if possible) counted by a different person.

Technical details should be reported on the reporting form of technical data.

Count results should be reported per type of RM on the relevant reporting form of count results and in the relevant Excel sheet. This double way of reporting data is for quality control of the data fed into the Excel sheet.

Information per method

ISO 6222 (1999). Water quality – Enumeration of culturable micro-organisms – Colony count by inoculation in a nutrient agar culture medium

The RMs are analysed by preparing pour-plates with Yeast extract agar. Of each RM, 5 units are analysed in duplicate for each incubation temperature. Follow the instructions of the ISO. Mind: Before pouring molten agar to the plates, measure the temperature of the molten agar in a control flask (or in another appropriate way) and note on the reporting form

For the analyses of the capsules, 5 capsules are reconstituted in 10 ml peptone saline solution (see SOP BCR-Water/001). Of each solution four times 1 ml is mixed with molten Yeast extract agar (see ISO 6222). After solidification, two plates of each capsule solution are incubated at (36 ± 2) °C for (44 ± 4) h and the two other plates are incubated at (22 ± 2) °C for (68 ± 4) h. Count the plates in a random order following the instruction of ISO 6222.

ISO/WD 6461-2 (2001). Water quality – Detection and enumeration of *Clostridium perfringens* – Part 2: Method by membrane filtration

The RMs are analysed by means of membrane filtration (10 in singular for lenticules and pastilles; 5 in duplicate for capsules) on TSC-agar. No heat treatment should be applied. Follow the instructions for each RM and follow the instructions given in ISO/WD 6461-2.

The reported counts are the counts obtained from the filters (cfp/100 ml). Confirmation tests will not be necessary as the RMs consist of pure cultures. Report raw data (no mean results).

ISO 7899-1 (1998). Water quality – Detection and enumeration of intestinal enterococci in surface and wastewater – Part 1: Miniaturized method (Most Probable Number) by inoculation in liquid medium

The RMs are analysed by means of a Most Probable Number method in a microtiter plate (10 in singular for lenticules and pastilles; 5 in duplicate for capsules). Consider the RM-solutions as fresh waters. Prepare dilutions as indicated for bathing water in ISO 7899-1 (64 wells with dilution 1/2 , 32 wells with dilution 1/20). Count the number of fluorescent (positive) wells of each dilution and calculate for each microtiter plate the MPN/100 ml. Report the number of positive wells as well as the MPN/100 ml, for each sample analysed (do not give mean results).

ISO 7899-2 (2000). Water quality – Detection and enumeration of intestinal enterococci – Part 2: Membrane filtration method

The RMs are analysed by means of membrane filtration (10 in singular for lenticules and pastilles; 5 in duplicate for capsules) on Slanetz and Bartley medium. Follow the instructions for each RM and follow the instructions given in ISO 7899-2.

The reported counts are the counts obtained from the filters (cfp/100 ml). Confirmation tests will not be necessary as the RMs consist of pure cultures. Report raw data (no mean results).

ISO 9308-1 (2000). Water quality – Detection and enumeration of *Escherichia coli* and coliform bacteria – Part 1: Membrane filtration method

The RMs are analysed by means of membrane filtration (10 in singular for lenticules and pastilles; 5 in duplicate for capsules) on Lactose TTC agar with sodium heptadecylsulfate. Follow the instructions for each RM and follow the instructions given in ISO 9308-1.

The reported counts are the counts obtained from the filters (cfp/100 ml). Confirmation tests will not be necessary as the RMs consist of pure cultures. Report raw data (no mean results).

ISO 9308-3 (1998). Water quality – Detection and enumeration of *Escherichia coli* and coliform bacteria in surface and wastewater – Part 3: Miniaturized method (Most Probable Number) by inoculation in liquid medium

The RMs are analysed by means of a Most Probable Number method in a microtiter plate (10 in singular for lenticules and pastilles; 5 in duplicate for capsules). Consider the RM-solutions as fresh waters. Prepare dilutions as indicated for bathing water in ISO 9308-3 (64 wells with dilution 1/2 , 32 wells with dilution 1/20). Count the number of fluorescent (positive) wells of each dilution and calculate for each microtiter plate the MPN/100 ml. Report the number of positive wells as well as the MPN/100 ml, for each sample analysed (do not give mean results).

prEN 12780 (1999). Water quality – Detection and enumeration of *Pseudomonas aeruginosa* by membrane filtration

The RMs are analysed by means of membrane filtration (10 in singular for lenticules and pastilles; no capsules are available for this type of RM) on CN-agar. Follow the instructions for each RM and follow the instructions given in prEN 12780.
The reported counts are the counts obtained from the filters (cfp/100 ml). Confirmation tests will not be necessary as the RMs consist of pure cultures. Report raw data (no mean results).

REPORTING OF THE RESULTS

Note the data per type of RM on the relevant reporting form of count results and send these forms by fax or normal mail (before 5 July 2001) to the contact persons as indicated on the last pages of the reporting forms. Keep a copy for your own information.

Enter the data in the relevant Excel sheets (separate for lenticules, pastilles and capsules). Send the complete Excel sheet **before 5 July 2002** by e-mail to the following persons:

- Data on lenticules to Dave Stewardson (University-Newcastle): D.J.Stewardson@ncl.ac.uk
- Data on pastilles to Tristan Simonart (IPL-Lille): tristan.simonart@pasteur-lille.fr;
- Data on capsules to Kirsten Mooijman(RIVM/MGB-Bilthoven): kirsten.mooijman@rivm.nl.

Send the completed reporting form of technical data by fax, e-mail or by normal mail to (keep a copy for your own information):

Kirsten Mooijman:

kirsten.mooijman@rivm.nl;

RIVM/MGB (Pb63); P.O.Box 1; 3720 BA Bilthoven, the Netherlands

fax: +31 30 274 4434

REFERENCES

ISO 6222 (1999). Water quality – Enumeration of culturable micro-organisms – Colony count by inoculation in a nutrient agar culture medium. International Organisation for Standardisation, Geneva, Switzerland.

ISO/WD 6461-2 (2001). Water quality – Detection and enumeration of *Clostridium perfringens* – Part 2: Method by membrane filtration. International Organisation for Standardisation, Geneva, Switzerland.

ISO 7899-1 (1998). Water quality – Detection and enumeration of intestinal enterococci in surface and wastewater – Part 1: Miniaturized method (Most Probable Number) by inoculation in liquid medium. International Organisation for Standardisation, Geneva, Switzerland.

ISO 7899-2 (2000). Water quality – Detection and enumeration of intestinal enterococci – Part 2: Membrane filtration method. International Organisation for Standardisation, Geneva, Switzerland.

ISO 9308-1 (2000). Water quality – Detection and enumeration of *Escherichia coli* and coliform bacteria – Part 1: Membrane filtration method. International Organisation for Standardisation, Geneva, Switzerland.

ISO 9308-3 (1998). Water quality – Detection and enumeration of *Escherichia coli* and coliform bacteria in surface and wastewater – Part 3: Miniaturized method (Most Probable Number) by inoculation in liquid medium. International Organisation for Standardisation, Geneva, Switzerland.

prEN 12780 (1999). Water quality – Detection and enumeration of *Pseudomonas aeruginosa* by membrane filtration. European Committee for Standardization, Brussels, Belgium.

Annex 2 SOP BCR-water/001

SOP BCR-water/001
08-03-2002

RIVM

RECONSTITUTION OF MICROBIOLOGICAL REFERENCE MATERIALS, CONSISTING OF GELATIN CAPSULES, IN 10 ml SOLUTION

1. INTRODUCTION

Relatively stable reference materials for quality control in water and food microbiology have been developed by the National Institute of Public Health and the Environment (RIVM), Bilthoven, The Netherlands. They consist of gelatin capsules filled with spray-dried milk, which has been artificially contaminated with a known test strain. A reconstitution procedure is described in this document in order to use the reference material for enumeration procedures. The result of this procedure will be a solution of *ca* 10 ml volume, that can be analysed by conventional enrichment, membrane filtration, pour plate or plate count procedures. Careful observation of all experimental details is required in order to assure reproducible results.

2. SCOPE AND FIELD OF APPLICATION

This standard operating procedure (SOP) describes a procedure for the reconstitution of reference materials as supplied by the National Institute of Public Health and the Environment (RIVM), Bilthoven, The Netherlands.

3. DEFINITION

For the purpose of this SOP the following definition applies.

Reference material: a gelatin capsule containing a measured amount of artificially contaminated spray dried milk.

4. PRINCIPLE

The reconstitution of reference materials involves two stages:

- Dissolution of the gelatin capsule in 10 ml peptone-saline solution at (38.5 ± 0.5) °C;
- Cooling of the solution in melting ice.

General: Unless otherwise stated, the tolerance interval of any measured value in this SOP is: stated value ± 5%.

5. CULTURE MEDIA

5.1 Basic materials

Use only distilled or deionized water that does not contain substances that might inhibit the growth of bacterial test strains in subsequent tests.

5.2 Reconstitution medium (peptone-saline solution)

Composition

Peptone	0.2 g
Sodium chloride (NaCl) p.a.	1.7 g
Water	200 ml

Preparation

Suspend the ingredients in the water. Dissolve, when necessary by heating, with frequent stirring. Transfer to 250 ml glass bottles. The pH should be 7.0 ± 0.5; adjust with 1 mol/l HCl or NaOH-solution when necessary. Sterilize by autoclaving at (121 ± 1) °C for (15 ± 1) min.

6. APPARATUS AND GLASSWARE

6.1 Apparatus

6.1.1 Waterbath, thermostatically controlled at (38.5 ± 0.5) °C.
6.1.2 Calibrated thermometer, traceable to primary standards, range 0-60 °C or another appropriate range, scale division 0.1 °C.
6.1.3 Sterile forceps with rounded edges for manupulating gelatin capsules.
6.1.4 Whirlmixer.
6.1.5 Stopwatch (minimal 60 minutes).

6.2 Glassware

6.2.1 Test tubes preferably of 25 mm diameter x 190 mm length (sterile), otherwise of 14-18 diameter x 160 mm length, closed with caps (not cotton plugs).
6.2.2 Pipettes or dispensor of 10 ml nominal capacity (sterile).
6.2.3 Glass bottles of 250 ml nominal capacity.
6.2.4 Glass beads with a diameter of *ca* 0.3 cm (sterile).

It is also possible to add 10-15 glass beads to the test tubes before sterilization.

7. PROCEDURE

Fill the test tubes (6.2.1) with (10.0 ± 0.2) ml peptone-saline solution of room temperature and 10-15 glass beads (6.2.4).
Place the tubes in the waterbath (6.1.1) for at least 30 min. Control the temperature in a reference tube with (10.0 ± 0.2) ml peptone-saline. When the temperature in the reference tube is constant (38.5 ± 0.5) °C, add one gelatin capsule (directly from the freezer) to each test tube (except the reference tube), preferably without taking the tubes from the waterbath.
Ten minutes after adding the gelatin capsules, place the tubes on the whirlmixer (6.1.4) and mix well, for 10-15 seconds. Control the time with the stopwatch (6.1.5). Avoid, by adjusting the mixing speed, formation of excessive foam. Replace the tubes in the waterbath.
Mix again after 20, 30 and 40 minutes as described above.
After the last mixing, cool the tubes in melting ice for at least 15 minutes and use the same day, leaving the tubes in melting ice.

Note: In order to assure good dissolution of the gelatin capsule it is of critical importance that:

- The temperature of the peptone-saline will not drop below 37 °C during the reconstitution procedure;
- The time interval during which the tube is outside the waterbath, must be kept as short as possible. Therefore, if a series of tubes is used in parallel, each tube should be taken out of the waterbath separately and replaced before another tube is taken.

Note: Make sure that the total reconstitution time (time between addition of the first capsule to peptone-saline and placing the last tube in melting ice) will not last longer than 50 minutes.

8. TEST REPORT

The test report should contain all information on operational details, not mentioned or specified in this SOP, that might influence the test result. Any incidents or deviations from the specifications should also be recorded.

Annex 3 RIVM/MGB-I001

RIVM/MGB-I001
14-03-2002

RIVM

INSTRUCTIONS FOR ANALYSING MICROBIOLOGICAL REFERENCE MATERIALS, CONSISTING OF GELATIN CAPSULES, WITH DIFFERENT METHODS

1. INTRODUCTION

Microbiological reference materials as supplied by the Microbiological Laboratory for Health Protection (MGB) of the National Institute of Public Health and the Environment (RIVM, Bilthoven, the Netherlands) consist of gelatin capsules. The capsules are filled with spray-dried milk, which has been artificially contaminated with a known test strain. To remain the materials stable they need to be stored at (-20 ± 5) °C. To make them ready for use for the studies of the MICROCRM project, a reconstitution procedure and instructions for use need to be followed. Reconstitution of the gelatin capsules is described in SOP BCR-water/001. Instructions for use are given below.

2. GENERAL

At the day of analyses, reconstitute the relevant number of capsules (see Protocol 'Feasibility certification studies of microbiological reference materials') according to SOP BCR-water/001.
Preparation of Peptone saline solution (PS) is described in SOP BCR-water/001.

3. INSTRUCTIONS FOR USE PER METHOD

3.1 ISO 6222 (1999). Water quality – Enumeration of culturable micro-organisms – Colony count by inoculation in a nutrient agar culture medium

In this ISO procedure separate counts are made of the micro-organisms which are able to grow and form colonies on nutrient agar media at 36 °C and at 22 °C. Both procedures (culturing at 36 °C and at 22 °C) will be perfromed with gelatin capsules containing *Enterococcus faecium* WR63 (NCTC 13160), batch LWL34-240701, in the following way:

- After cooling of the reconstitution mixture in melting ice, the contents of the tubes are mixed on a whirlmixer;
- Analyse (1.00 ± 0.02) ml of the reconstitution solution according to ISO 6222 and incubate at 22 °C or at 36 °C.

3.2 ISO/WD 6461-2 (2001). Water quality – Detection and enumeration of *Clostridium perfringens* – Part 2: Method by membrane filtration

The procedure described in ISO/WD 6461-2 will be performed with gelatin capsules containing *Clostridium perfringens* D10 (NCTC 13170), batch LWL3501 241001 in the following way:

- After cooling of the reconstitution mixture in melting ice, the contents of the tubes are mixed on a whirlmixer;
- Place a membrane filter in a filtration funnel. Add approximately 100 ml peptone saline solution (PS) of room temperature to the filtration funnel.
  Add (1.00 ± 0.02) ml capsule solution to the PS. Mix well. If it is not possible to mix the filtration funnel (e.g. in case it is fixed on the laboratory bench) add the 1.00 ml capsule solution to 80 ml PS (room temperature) in the filtration funnel and pour another 20 ml PS (room temperature) to the contents of the filtration funnel. Filter through the membrane filter. Using sterile forceps, place the membrane filter on the agar medium of room temperature and incubate according to the procedure as described in ISO/WD 6461-2.

3.3 ISO 7899-1 (1998). Water quality – Detection and enumeration of intestinal enterococci in surface and waste water – Part 1: Miniaturized method (Most Probable Number) by inoculation in liquid medium

The procedure described in ISO 7899-1 will be performed with gelatin capsules containing *Enterococcus faecium* WR63 (NCTC 13160), batch LWL34-240701, in the following way:

- After cooling of the reconstitution mixture in melting ice, the contents of the tubes are mixed on a whirlmixer;
- Take (3.00 ± 0.06) ml of the reconstitution mixture and add to 100 ml cool (*ca* 5 °C) peptone saline solution (PS). Mix well. Analyse this mixture in accordance with ISO 7899-1, considering the sample as bathing water.

3.4 ISO 7899-2 (2000). Water quality – Detection and enumeration of intestinal enterococci – Part 2: Membrane filtration method

The procedure described in ISO 7899-2 will be performed with gelatin capsules containing *Enterococcus faecium* WR63 (NCTC 13160), batch LWL34-240701, in the following way:

- After cooling of the reconstitution mixture in melting ice, the contents of the tubes are mixed on a whirlmixer;
- Place a membrane filter in a filtration funnel. Add approximately 100 ml peptone saline solution (PS) of room temperature to the filtration funnel.
  Add (1.00 ± 0.02) ml capsule solution to the PS. Mix well. If it is not possible to mix the filtration funnel (e.g. in case it is fixed on the laboratory bench) add the 1 ml capsule solution to 80 ml PS (room temperature) in the filtration funnel and pour another 20 ml PS (room temperature) to the contents of the filtration funnel. Filter through the membrane filter. Using sterile forceps, place the membrane filter on the agar medium of room temperature and incubate according to the procedure as described in ISO 7899-2.

3.5 ISO 9308-1 (2000). Water quality – Detection and enumeration of *Escherichia coli* and coliform bacteria – Part 1: Membrane filtration method

The procedure described in ISO 9308-1 will be performed with gelatin capsules containing *Escherichia coli* WR1 (NCTC 13167), batch 6-2 250601, in the following way:

- After cooling of the reconstitution mixture in melting ice, the contents of the tubes are mixed on a whirlmixer;
- Place a membrane filter in a filtration funnel. Add approximately 100 ml peptone saline solution (PS) of room temperature to the filtration funnel.
  Add (0.50 ± 0.01) ml capsule solution to the PS. Mix well. If it is not possible to mix the filtration funnel (e.g. in case it is fixed on the laboratory bench) add the 0.5 ml capsule solution to 80 ml PS (room temperature) in the filtration funnel and pour another 20 ml PS (room temperature) to the contents of the filtration funnel. Filter through the membrane filter. Using sterile forceps, place the membrane filter on the agar medium of room temperature and incubate according to the procedure as described in ISO 9308-1.

3.6 ISO 9308-3 (1998). Water quality – Detection and enumeration of *Escherichia coli* and coliform bacteria in surface and waste water – Part 3: Miniaturized method (Most Probable Number) by inoculation in liquid medium

The procedure described in ISO 9308-3 will be performed with gelatin capsules containing *Escherichia coli* WR1 (NCTC 13167), batch 6-2 250601, in the following way:

- After cooling of the reconstitution mixture in melting ice, the contents of the tubes are mixed on a whirlmixer;
- Take (4.00 ± 0.08) ml of the reconstitution mixture and add to 100 ml cool (*ca* 5 °C) peptone saline solution (PS). Mix well. Analyse this mixture in accordance with ISO 7899-1, considering the sample as bathing water.

3.7 prEN 12780 (1999). Water quality – Detection and enumeration of *Pseudomonas aeruginosa* by membrane filtration

No stable capsules available

References

SOP BCR-water/001 (08-03-2002). Reconstitution of microbiological reference materials, consisting of gelatin capsules, in 10 ml solution. RIVM.

ISO 6222 (1999). Water quality – Enumeration of culturable micro-organisms – Colony count by inoculation in a nutrient agar culture medium. International Organisation for Standardisation, Geneva, Switzerland.

ISO/WD 6461-2 (2001). Water quality – Detection and enumeration of *Clostridium perfringens* – Part 2: Method by membrane filtration. International Organisation for Standardisation, Geneva, Switzerland.

ISO 7899-1 (1998). Water quality – Detection and enumeration of intestinal enterococci in surface and waste water – Part 1: Miniaturized method (Most Probable Number) by inoculation in liquid medium. International Organisation for Standardisation, Geneva, Switzerland.

ISO 7899-2 (2000). Water quality – Detection and enumeration of intestinal enterococci – Part 2: Membrane filtration method. International Organisation for Standardisation, Geneva, Switzerland.

ISO 9308-1 (2000). Water quality – Detection and enumeration of *Escherichia coli* and coliform bacteria – Part 1: Membrane filtration method. International Organisation for Standardisation, Geneva, Switzerland.

ISO 9308-3 (1998). Water quality – Detection and enumeration of *Escherichia coli* and coliform bacteria in surface and waste water – Part 3: Miniaturized method (Most Probable Number) by inoculation in liquid medium. International Organisation for Standardisation, Geneva, Switzerland.

prEN 12780 (1999). Water quality – Detection and enumeration of *Pseudomonas aeruginosa* by membrane filtration. European Committee for Standardization, Brussels, Belgium.

Annex 4 Lenticules-I002

Lenticules-I002
08-03-2002

PHLS

REHYDRATION AND PREPARATION OF LENTICULE™ DISCS FOR USE.

Remove tube(s) to be used from freezer storage at -20°C ± 5°C.
Do not loosen or remove top(s) of tube(s).
Leave to stand at room temperature for 10 min (± 1 min) to ensure contents of the tube(s) come up to ambient temperature.

Remove top from tube. Each tube contains a coloured LENTICULE™ disc, lens shaped in appearance, resting on a filter support containing self-indicating silica gel. Ensure that the disc is on top of the filter support. If the disc has slipped down between the filter and the side of the tube, ease out gently using fine forceps and place on top of the filter.

Invert tube and tip disc into sterile 0.1% peptone saline solution (also called maximum recovery diluent, MRD; for preparation see SOP BCR-water/001, 5.2), ensuring that the LENTICULE ™ disc falls into the peptone saline solution. It may float or sink. Replace the top loosely to the bottle (do not yet tighten).
If the disc gets stuck on the side of the bottle, carefully tighten the top and place bottle in such a position that the disc is immersed in the peptone saline solution.

Volumes of peptone saline solution to be used:
- For membrane filtration: 100 ml (±1 ml)
- For pour plate colony counts: 2.5 ml (± 0.25 ml).

Note: The peptone saline solution should be at ambient temperature before use.

Leave to stand for 10 min (± 1 min). Do not tighten top of bottle or shake/agitate at this stage.

Tighten the top of the bottle and mix:
- Shaking 100 ml volumes vigorously by inverting the bottle 30 times in 15 s (± 5 s)
- Vortexing 2.5 ml volumes for 15 s (± 5 s).

During the mixing process froth is generated and, to allow break down of the froth, leave to stand after mixing:
- 10 min (± 1 min) for 100 ml volumes
- 1 min for 2.5 ml volumes.

The prepared solution is now ready for use.
It is recommended that it should be used within 30 min (± 5 min) for enumeration procedures.

TM: LENTICULE™ is a trademark of The Public Health Laboratory Service Board.

Annex 5 SOP IPL/002

SOP IPL/002
15-03-2002

IPL

REHYDRATION AND PREPARATION OF PASTILLES FOR USE

1. INTRODUCTION

Relatively stable reference materials for quality control in water and food microbiology have been developed by the Institut Pasteur de Lille (IPL; France). They consist of dehydrated pastilles containing a known test strain. A reconstitution procedure is described in this document in order to use the reference material for enumeration procedures. The result of this procedure will be a solution that can be analysed by conventional enrichment, membrane filtration, pour plate or plate count procedures. Careful observation of all experimental details is required in order to assure reproducible results.

2. SCOPE AND FIELD OF APPLICATION

This standard procedure describes a procedure for the reconstitution of pastilles (stored in individual vials at –20°C ± 5°C) as supplied by IPL, France.

3. DEFINITION

N/A

4. PRINCIPLE

The reconstitution of pastilles involves a dissolution of the pastille in a peptone-saline solution at room temperature (20°C ± 5°C)

General: Unless otherwise stated, the tolerance interval of any measured value in this SOP is: stated value ± 5%.

5. CULTURE MEDIA

5.1 Basic materials

Use only distilled or deionized water that does not contain substances that might inhibit the growth of bacterial test strains in subsequent tests.

5.2 Reconstitution medium (peptone-saline solution (PS))

Composition

Peptone	0.2 g
Sodium chloride (NaCl) p.a.	1.7 g
Water	200 ml

Preparation

Suspend the ingredients in the water. Dissolve, when necessary by heating, with frequent stirring. Transfer in volumes of 100 ml ± 1 ml to (*ca*) 150 ml glass bottles. The pH should be 7.0 ± 0.5; adjust with 1 mol/l HCl or NaOH-solution when necessary. Sterilize by autoclaving at (121 ± 1) °C for (15 ± 1) min.

6. APPARATUS AND GLASSWARE

6.1 Apparatus

6.1.1 Sterile fine forceps for manipulating pastilles (if necessary).
6.1.4 Whirlmixer.

6.2 Glassware

6.2.1 Test tubes of 12-18 diameter x 120-160 mm length, closed with caps (not cotton plugs).
6.2.2 Pipettes or dispensor of 10 ml nominal capacity (sterile).
6.2.3 Glass bottles of (*ca*) 150 ml nominal capacity.

7. PROCEDURE

- Remove pastille containing-vial(s) to be used from freezer storage at -20°C ± 5°C;
- Leave to stand for 10 min (± 5 min) to reach ambient temperature;
- Remove top from vial(s), invert and tip pastille into sterile peptone saline diluent (PS):
- Use 10 ml ± 0.2 ml sterile PS in a tube for culturable organisms (ISO 6222);
- Use 100 ml ± 1ml sterile PS in a bottle for the other organisms (filtration and MPN methods)

See also Table A.5.1.

- Leave bottle or tube to stand for 2 min (± 1 min).
- Shake bottles (containing 100 ml solutions)10 times in 15 sec (± 5 sec)
- Vortex tubes (containing 10 ml solutions) for 15 sec (± 5 sec).
- Keep bottle or tube preferably in melting ice or alternatively at room temperature.
- Use within 30 min after finalising the reconstitution procedure. For the enumeration procedures see Table 1 and instructions for use in document *Pastilles-I003*, *15-03-2002*.

Table A.5.1 List of supplied pastilles, analytical methods, PS rehydration volume for standard sample and sample volume to be analysed

Pastille batch number and target organism	Analytical method	Volume of PS to be used for reconstitution of standard sample	Standard sample volume to be analysed
MICRO-CRM-EC1 *Escherichia coli*	ISO 9308-1	100 ml ± 1 ml bottle	100 ml ± 1 ml
MICRO-CRM-IE1 Intestinal enterococci	ISO 7899-2	100 ml ± 1 ml bottle	100 ml ± 1 ml
MICRO-CRM-C1 *Clostridium perfringens*	ISO(WD) 6461-2 without heating	100 ml ± 1 ml bottle	100 ml ± 1 ml
MICRO-CRM-GV22 Culturable organisms (22°C)	ISO 6222	10 ± 0.2 ml tube	1 ml ± 0.05 ml
MICRO-CRM-GV36 Culturable organisms (36°C)	ISO 6222	10 ± 0.2 ml tube	1 ml ± 0.05 ml
MICRO-CRM-PA *Pseudomonas aeruginosa*	(pr)EN 12780	100 ml ± 1 ml bottle	**50 ml ± 0.5 ml**
MICRO-CRM-EC2 *Escherichia coli*	ISO 9308-3 (MPN)	100 ml ± 1 ml bottle	1/2 & 1/20 dilutions
MICRO-CRM-IE2 Intestinal enterococci	ISO 7899-1 (MPN)	100 ml ± 1 ml bottle	1/2 & 1/20 dilutions

8. TEST REPORT

The test report should contain all information on operational details, not mentioned or specified in this procedure, that might influence the test result. Any incidents or deviations from the specifications should also be recorded.

Annex 6 Pastilles-I003

Pastilles-I003
15-03-2002

IPL

INSTRUCTIONS FOR ANALYSING MICROBIOLOGICAL REFERENCE MATERIALS, CONSISTING OF DEHYDRATED PASTILLES, WITH DIFFERENT METHODS

1. INTRODUCTION

Microbiological Reference Materials (RM) as supplied by the Institut Pasteur de Lille (IPL, France) consist of dehydrated pastilles. The pastilles contains a known test strain in a known concentration related to a standard analytical method. To remain the materials stable they need to be stored at (-20 ± 5) °C. To make them ready for use for the studies of the MICROCRM project, a reconstitution procedure (see SOP IPL/002) and instructions for use (described in this document Pastilles-I003) need to be followed.

2. GENERAL

At the day of analyses, reconstitute the relevant number of pastilles (see Protocol 'Feasibility certification studies of microbiological reference materials') according to SOP IPL/002.

3. INSTRUCTIONS FOR USE PER METHOD

3.1 **ISO 6222 (1999). Water quality – Enumeration of culturable micro-organisms – Colony count by inoculation in a nutrient agar culture medium**

In this ISO procedure separate counts are made of the micro-organisms which are able to grow and form colonies on nutrient agar media at 36 °C and at 22 °C.

Procedures (culturing at 36 °C and at 22 °C) will be performed with pastilles:
- batch MICRO-CRM-GV22 for culturing at 22 °C and
- batch MICRO-CRM-GV36 for culturing at 36 °C

in the following way:
- After preparation of standard samples in 10 ml sterile peptone saline (in tubes), the contents of each tube is mixed on a whirlmixer;
- Analyse (1.00 ± 0.05) ml of the reconstitution solution according to ISO 6222 and incubate at 22 °C or at 36 °C.

3.2 ISO/WD 6461-2 (2001). Water quality – Detection and enumeration of *Clostridium perfringens* – Part 2: Method by membrane filtration

The procedure described in ISO/WD 6461-2 will be performed with batch of pastilles MICRO-CRM-C1 in the following way:
- Prepare standard samples in 100 ml peptone saline (in bottles);
- Place a membrane filter in a filtration funnel. Add the content of the bottle (100 ml) into the filtration funnel;
- Filter through the membrane filter. Using sterile forceps, place the membrane filter on the agar medium of room temperature and incubate according to the procedure as described in ISO/WD 6461-2.

3.3 ISO 7899-1 (1998). Water quality – Detection and enumeration of intestinal enterococci in surface and waste water – Part 1: Miniaturized method (Most Probable Number) by inoculation in liquid medium

The procedure described in ISO 7899-1 will be performed with batch of pastilles MICRO-CRM-IE2, in the following way:
- After preparation of standard samples in 100 ml peptone saline (in bottles), analyse the mixtures in accordance with ISO 7899-1, considering the samples as fresh bathing water (peptone saline water salinity < 30g/l): both 1/2 and 1/20 dilutions in special diluent.

3.4 ISO 7899-2 (2000). Water quality – Detection and enumeration of intestinal enterococci – Part 2: Membrane filtration method

The procedure described in ISO 7899-2 will be performed with batch of pastilles MICRO-CRM-IE1, in the following way:
- Prepare standard samples in 100 ml peptone saline (in bottles);
- Place a membrane filter in a filtration funnel. Add the content of the bottle (100 ml) into the filtration funnel;
- Filter through the membrane filter. Using sterile forceps, place the membrane filter on the agar medium of room temperature and incubate according to the procedure as described in ISO 7899-2.

3.5 ISO 9308-1 (2000). Water quality – Detection and enumeration of *Escherichia coli* and coliform bacteria – Part 1: Membrane filtration method

The procedure described in ISO 9308-1 will be performed with batch of pastilles MICRO-CRM-EC1 in the following way:
- Prepare standard samples in 100 ml peptone saline (in bottles);
- Place a membrane filter in a filtration funnel. Add the content of the bottle (100 ml) into the filtration funnel;
- Filter through the membrane filter. Using sterile forceps, place the membrane filter on the agar medium of room temperature and incubate according to the procedure as described in ISO 9308-1.

3.6 ISO 9308-3 (1998). Water quality – Detection and enumeration of *Escherichia coli* and coliform bacteria in surface and waste water – Part 3: Miniaturized method (Most Probable Number) by inoculation in liquid medium

The procedure described in ISO 9308-3 will be performed with batch of pastilles MICRO-CRM-EC2, in the following way:

- After preparation of standard samples in 100 ml peptone saline (in bottles), analyse the mixtures in accordance with ISO 9308-3**,** considering the samples as fresh bathing water (peptone saline water salinity < 30g/l): both 1/2 and 1/20 dilutions in special diluent.

3.7 prEN 12780 (November 1999). Water quality – Detection and enumeration of *Pseudomonas aeruginosa* by membrane filtration.

The procedure described in prEN 12780 will be performed with batch of pastilles MICRO-CRM-PA, in the following way:

- Prepare standard samples in 100 ml peptone saline (in bottles);
- Place a membrane filter in a filtration funnel. From the standard sample bottle, add **50 ml ± 0.5 ml** (attention: do not add 100 ml) to the filtration funnel.
- Filter through the membrane filter. Using sterile forceps, place the membrane filter on the agar medium of room temperature and incubate according to the procedure as described in prEN 12780.

References

SOP IPL/002 (15-03-2002). Rehydration and preparation of pastilles for use. Institut Pasteur de Lille, Water and Environment Department, Lille, France.

ISO 6222 (1999). Water quality – Enumeration of culturable micro-organisms – Colony count by inoculation in a nutrient agar culture medium. International Organisation for Standardisation, Geneva, Switzerland.

ISO/WD 6461-2 (2001). Water quality – Detection and enumeration of *Clostridium perfringens* – Part 2: Method by membrane filtration. International Organisation for Standardisation, Geneva, Switzerland.

ISO 7899-1 (1998). Water quality – Detection and enumeration of intestinal enterococci in surface and waste water – Part 1: Miniaturized method (Most Probable Number) by inoculation in liquid medium. International Organisation for Standardisation, Geneva, Switzerland.

ISO 7899-2 (2000). Water quality – Detection and enumeration of intestinal enterococci – Part 2: Membrane filtration method. International Organisation for Standardisation, Geneva, Switzerland.

ISO 9308-1 (2000). Water quality – Detection and enumeration of *Escherichia coli* and coliform bacteria – Part 1: Membrane filtration method. International Organisation for Standardisation, Geneva, Switzerland.

ISO 9308-3 (1998). Water quality – Detection and enumeration of *Escherichia coli* and coliform bacteria in surface and waste water – Part 3: Miniaturized method (Most Probable Number) by inoculation in liquid medium. International Organisation for Standardisation, Geneva, Switzerland.

prEN 12780 (November 1999). Water quality – Detection and enumeration of *Pseudomonas aeruginosa* by membrane filtration. European Committee for Standardization, Brussels, Belgium.

Annex 7 Pastilles-I004

Pastilles-I004
15-04-2002

IPL

INSTRUCTIONS FOR QUANTITATIVE QUALITY CONTROL OF CULTURE MEDIA USING PASTILLES (REFERENCE MATERIALS)

1. INTRODUCTION

Microbiological Reference Materials (RM) as supplied by the Institut Pasteur de Lille (IPL, France) consist of dehydrated pastilles. The pastilles contains a known test strain in a known concentration related to a standard analytical method. To remain the materials stable they need to be stored at (-20 ± 5) °C. To make them ready for use for the quality control of culture media , the following instructions for use (described in this document Pastilles-I004) need to be followed.

2. SCOPE AND FIELD OF APPLICATION

This standard procedure describes a procedure for the quality control of culture media using pastilles (each vial contains 5 pastilles and must be stored at –20°C ± 5°C) as supplied by IPL, France.

3. DEFINITION

N/A

4. PRINCIPLE

The quality control of culture media involves a reconstitution of pastilles after dissolution in a peptone-saline solution at room temperature (20°C ± 5°C)

5. MATERIALS

5.1 Basic materials

Use only distilled or deionized water that does not contain substances that might inhibit the growth of bacterial test strains in subsequent tests.

5.2 Reconstitution medium (peptone-saline solution (PS))

Composition

Peptone	0.2 g
Sodium chloride (NaCl) p.a.	1.7 g
Water	200 ml

Preparation
Suspend the ingredients in the water. Dissolve, when necessary by heating, with frequent stirring. Transfer in volumes of 100 ml ± 1 ml to (*ca*) 150 ml glass bottles. The pH should be 7.0 ± 0.5; adjust with 1 mol/l HCl or NaOH-solution when necessary. Sterilize by autoclaving at (121 ± 1) °C for (15 ± 1) min.

6. APPARATUS AND GLASSWARE

6.1 Apparatus

6.1.1 Sterile fine forceps for manipulating pastilles (if necessary).
6.1.4 Whirlmixer.

6.2 Glassware

6.2.1 Test tubes of 12-18 diameter x 120-160 mm length, closed with caps (not cotton plugs).
6.2.4 Pipettes or dispensor of 10 ml nominal capacity (sterile).
6.2.5 Glass bottles of (*ca*) 150 ml nominal capacity.

7. PROCEDURE

- Remove pastille containing-vial(s) to be used from freezer storage at -20°C ± 5°C;
- Leave to stand for 10 min (± 5 min) to reach ambient temperature;
- Remove top from vial(s) and, using fine forceps, tip one pastille in each of the 5 tubes or bottle containing sterile peptone saline diluent (PS):
  - TUBE: Use 5 ml ± 0.2 ml sterile PS in a tube for culturable organisms (ISO 6222);
  - BOTTLE: Use 100 ml ± 1ml sterile PS in a bottle for the other organisms (filtration methods).

See also Table A.7.1.

- Leave bottle or tube to stand for 2 min (± 1 min).
- Shake bottles (containing 100 ml solutions)10 times in 15 sec (± 5 sec)
- Vortex tubes (containing 10 ml solutions) for 15 sec (± 5 sec).
- Keep bottle or tube preferably in melting ice or alternatively at room temperature.
- Use within 30 min after finalising the reconstitution procedure.

Note1: the incubation temperature for the quality control of *Yeast extract agar (YA)* is 36°C (not 22°C).

QUALITY CONTROL

Each batch of culture medium should be controlled using 5 control samples (5 tubes or 5 bottles containing 1 pastille). Counts must be reported and mean count calculated.

Mean count = (count1 + count2 + count 3 +count 4 + count 5) / 5

Mean count must be included within acceptable range (see table 1). If the mean value is outside acceptable range, a second control is acceptable. In case of failure after 2nd control , the batch of medium should not be used for the certification study. Reason for failure should be investigated and reported.

8. TEST REPORT

The test report should contain all information on operational details, not mentioned or specified in this procedure, that might influence the test result. Any incidents or deviations from the specifications should also be recorded.

Table A.7.2 or similar table might be used to report the results.

Table A.7.1 List of supplied pastilles, analytical methods, PS rehydration volume for standard sample and sample volume to be analysed

Pastille batch number and target organism	Analytical method	Name of culture medium	Volume of PS to be used for reconstitution of control sample	Dilution factor	Volume to be analysed for quality control	Target count	Acceptable limits (low and high mean values) (n=5 pastilles)
MICRO-CRM- IPL-376 Culturable organisms (36°C only)	ISO 6222	*Yeast extract agar (YA)*	5 ± 0.2 ml tube	1/5	**1 ml ± 0.05 ml**	**50 (at 36°C)**	**30 < mean count < 70**
MICRO-CRM-IPL-539 *Escherichia coli*	ISO 9308-1	*Lactose TTC agar with sodium heptadecylsulfate (LTTC) basal medium+ TTC solution + Tergitol 7*	100 ml ± 1 ml bottle	1/100	**1 ml ± 0.05 ml**	**59**	**20 < mean count < 120**
MICRO-CRM- IPL-435 Intestinal enterococci	ISO 7899-2	*Slanetz and Bartley (S&B) basal medium + TTCsolution*	100 ml ± 1 ml bottle	1/10	**10 ml ± 0.5 ml**	**44**	**20 < mean count < 70**
MICRO-CRM- IPL-555 *Clostridium perfringens*	ISO(WD) 6461-2 without heating	*TSC agar without egg yolk (TSC) basal medium + Cycloserine solution*	100 ml ± 1 ml bottle	1/5	**20 ml ± 1 ml**	**75**	**50 < mean count < 95**
MICRO-CRM- IPL-543 *Pseudomonas aeruginosa*	(pr)EN 12780	*Pseudomonas agar / CN-agar basal medium + CN supplement*	100 ml ± 1 ml bottle	1/10	**10 ml ± 0.5 ml**	**65**	**25 < mean count < 115**

Table A.7.2 Reporting table for quality control of batches of culture media

Method	*Name of culture medium*	**Culture medium batch number**	**Date of control**	**Batch of Pastilles used**	**Target count**	**Acceptable limits (low and high mean values)**	**Observed counts (n = 5 pastilles)**	**Batch validated (YES or NO)**
ISO 6222 (36°C)	*Yeast extract agar (YA)*			Batch MICROCRM-IPL-376 :CULTURABLE ORGANISMS(36°C)"	**50**	**30 < mean count < 70**	n1= n2= n3= n4= n5= mean count=	
				Batch MICROCRM-IPL-376 :CULTURABLE ORGANISMS(36°C)"	**50**	**30 < mean count < 70**	n1= n2= n3= n4= n5= mean count=	
				Batch MICROCRM-IPL-376 :CULTURABLE ORGANISMS(36°C)"	**50**	**30 < mean count < 70**	n1= n2= n3= n4= n5= mean count=	
				Batch MICROCRM-IPL-376 :CULTURABLE ORGANISMS(36°C)"	**50**	**30 < mean count < 70**	n1= n2= n3= n4= n5= mean count=	

Method	*Name of culture medium*	**Culture medium batch number**	**Date of control**	**Batch of Pastilles used**	**Target count**	**Acceptable limits (low and high mean values)**	**Observed counts (n = 5 pastilles)**	**Batch validated (YES or NO)**
ISO/WD 6461-2	*Tryptose Sulphite Cycloserine agar without egg yolk (TSC) basal medium + Cycloserine solution*			Batch MICROCRM-IPL- 555: CLOSTRIDIUM PERFRINGENS	**75**	**50 < mean count < 95**	n1= n2= n3= n4= n5= mean count=	
				Batch MICROCRM-IPL- 555: CLOSTRIDIUM PERFRINGENS	**75**	**50 < mean count < 95**	n1= n2= n3= n4= n5= mean count=	
				Batch MICROCRM-IPL- 555: CLOSTRIDIUM PERFRINGENS	**75**	**50 < mean count < 95**	n1= n2= n3= n4= n5= mean count=	
				Batch MICROCRM-IPL- 555: CLOSTRIDIUM PERFRINGENS	**75**	**50 < mean count < 95**	n1= n2= n3= n4= n5= mean count=	

Method	*Name of culture medium*	**Culture medium batch number**	**Date of control**	**Batch of Pastilles used**	**Target count**	**Acceptable limits (low and high mean values)**	**Observed counts (n = 5 pastilles)**	**Batch validated (YES or NO)**
ISO 7899-2	*Slanetz and Bartley (S&B) basal medium + TTCsolution*			Batch MICROCRM-IPL- 435: INTESTINAL ENTEROCOCCI	**44**	**20 < mean count < 70**	n1= n2= n3= n4= n5= mean count=	
				Batch MICROCRM-IPL- 435: INTESTINAL ENTEROCOCCI	**44**	**20 < mean count < 70**	n1= n2= n3= n4= n5= mean count=	
				Batch MICROCRM-IPL- 435: INTESTINAL ENTEROCOCCI	**44**	**20 < mean count < 70**	n1= n2= n3= n4= n5= mean count=	
				Batch MICROCRM-IPL- 435: INTESTINAL ENTEROCOCCI	**44**	**20 < mean count < 70**	n1= n2= n3= n4= n5= mean count=	

Method	Name of culture medium	**Culture medium batch number**	**Date of control**	**Batch of Pastilles used**	**Target count**	**Acceptable limits (low and high mean values)**	**Observed counts (n = 5 pastilles)**	**Batch validated (YES or NO)**
ISO 9308-1	*Lactose TTC agar with sodium heptadecylsulfate (LTTC) basal medium+ TTC solution + Tergitol 7*			Batch MICROCRM-IPL- 539: ESCHERICHIA COLI	**59**	**20 < mean count < 120**	n1= n2= n3= n4= n5= mean count=	
				Batch MICROCRM-IPL- 539: ESCHERICHIA COLI	**59**	**20 < mean count < 120**	n1= n2= n3= n4= n5= mean count=	
				Batch MICROCRM-IPL- 539: ESCHERICHIA COLI	**59**	**20 < mean count < 120**	n1= n2= n3= n4= n5= mean count=	
				Batch MICROCRM-IPL- 539: ESCHERICHIA COLI	**59**	**20 < mean count < 120**	n1= n2= n3= n4= n5= mean count=	

Method	*Name of culture medium*	**Culture medium batch number**	**Date of control**	**Batch of Pastilles used**	**Target count**	**Acceptable limits (low and high mean values)**	**Observed counts (n = 5 pastilles)**	**Batch validated (YES or NO)**
prEN 12780	*Pseudomonas agar / CN-agar basal medium + CN supplement*			Batch MICROCRM-IPL- 543: PSEUDOMONAS AERUGINOSA	**65**	**25 < mean count < 115**	n1= n2= n3= n4= n5= mean count=	
				Batch MICROCRM-IPL- 543: PSEUDOMONAS AERUGINOSA	**65**	**25 < mean count < 115**	n1= n2= n3= n4= n5= mean count=	
				Batch MICROCRM-IPL- 543: PSEUDOMONAS AERUGINOSA	**65**	**25 < mean count < 115**	n1= n2= n3= n4= n5= mean count=	
				Batch MICROCRM-IPL- 543: PSEUDOMONAS AERUGINOSA	**65**	**25 < mean count < 115**	n1= n2= n3= n4= n5= mean count=	

Annex 8 Reporting form technical data

14-03-2002

REPORTING FORM

TECHNICAL DATA OF THE
FEASIBILITY CERTIFICATION STUDIES OF MICROBIOLOGICAL REFERENCE MATERIALS

Laboratory name: .....

Labcode: .....

Contact person : .....

Shipment of Lenticules:

Date of arrival parcel: ..... - ..... – 2002 Parcel damaged: ☐ yes ☐ no

Date and time the lenticules were placed at (-20 ± 5) °C:

Date: ..... - ..... – 2002 Time: .....

Shipment of pastilles:

Date of arrival parcel: ..... - ..... – 2002 Parcel damaged: ☐ yes ☐ no

Date and time the pastilles were placed at (-20 ± 5) °C:

Date: ..... - ..... – 2002 Time: .....

Shipment of capsules:

Date of arrival parcel: ..... - ..... – 2002 Parcel damaged: ☐ yes ☐ no

Date and time the capsules were placed at (-20 ± 5) °C:

Date: ..... - ..... – 2002 Time: .....

DATES

1. Give the dates of analyses for each method and each type of RM.

	Lenticules	Pastilles	Capsules
ISO 6222; 22 °C			
ISO 6222; 36 °C			
ISO/WD 6461-2			
ISO 7899-1 (MPN)			
ISO 7899-2 (mf)			
ISO 9308-1 (mf)			
ISO 9308-3 (MPN)			
prEN 12780			

REMARKS:

MEDIA AND FILTERS

2. Give information on media

General: If no 'ready-for-use' medium was used please indicate 'from components'.

2.1 ISO 6222

Yeast extract agar (YA):

Manufacturer: .....

Catalogue no. manufacturer: .....

Batch number manufacturer: .....

Date(s) of preparation: .....

Batch no. laboratory: .....

Temperature of storage (°C): .....

pH (and measuring temp.): pH:..... measuring temperature:..... °C

Quality control results: .....

.....

2.2 ISO/WD 6461-2

Tryptose Sulphite Cycloserine agar without egg yolk (TSC) basal medium:

Manufacturer: .....

Catalogue no. manufacturer: .....

Batch number manufacturer: .....

Date(s) of preparation: .....

Batch no. laboratory: .....

Temperature of storage (°C): .....

Cycloserine solution:

Manufacturer: .....

Catalogue no. manufacturer: .....

Batch number manufacturer: .....

Date(s) of preparation: .....

Batch no. laboratory: .....

Temperature of storage (°C): .....

Complete medium:

pH (and measuring temp.): pH:..... measuring temperature:..... °C

Quality control results: .....

2.3 ISO 7899-1

MUD/SF medium:

Manufacturer: .....

Catalogue no. manufacturer: .....

Batch number manufacturer: .....

Temperature of storage (°C): .....

If prepared from components:

Date(s) of preparation: .....

Batch no. laboratory: .....

pH (and measuring temp.): pH:..... measuring temperature:..... °C

Quality control results: .....

Special Diluent:

Manufacturer: .....

Catalogue no. manufacturer: .....

Batch number manufacturer: .....

Date(s) of preparation: .....

Batch no. laboratory: .....

Temperature of storage (°C): .....

pH (and measuring temp.): pH:..... measuring temperature:..... °C

Quality control results: .....

2.4 ISO 7899-2

Slanetz and Bartley (S&B) basal medium:

Manufacturer: .....

Catalogue no. manufacturer: .....

Batch number manufacturer: .....

Date(s) of preparation: .....

Batch no. laboratory: .....

Temperature of storage (°C): .....

TTC solution:

Manufacturer: .....

Catalogue no. manufacturer: .....

Batch number manufacturer: .....

Date(s) of preparation: .....

Batch no. laboratory: .....

Temperature of storage (°C): .....

Complete medium:

pH (and measuring temp.): pH:..... measuring temperature:..... °C

Quality control results: .....

2.5 ISO 9308-1

Lactose TTC agar with sodium heptadecylsulfate (LTTC) basal medium:

Manufacturer: .....

Catalogue no. manufacturer: .....

Batch number manufacturer: .....

Date(s) of preparation: .....

Batch no. laboratory: .....

Temperature of storage (°C): .....

pH (and measuring temp.): pH:..... measuring temperature:..... °C

TTC solution:

Manufacturer: .....

Catalogue no. manufacturer: .....

Batch number manufacturer: .....

Date(s) of preparation: .....

Batch no. laboratory: .....

Temperature of storage (°C): .....

Sodium heptadecylsulfate solution (Tergitol 7):

Manufacturer: .....

Catalogue no. manufacturer: .....

Batch number manufacturer: .....

Date(s) of preparation: .....

Batch no. laboratory: .....

Temperature of storage (°C): .....

Complete medium:

pH (and measuring temp.): pH:..... measuring temperature:..... °C

Quality control results: .....

2.6 ISO 9308-3

MUG/EC medium:

Manufacturer: .....

Catalogue no. manufacturer: .....

Batch number manufacturer: .....

Temperature of storage (°C): .....

If prepared from components:

Date(s) of preparation: .....

Batch no. laboratory: .....

pH (and measuring temp.): pH:..... measuring temperature:..... °C

Quality control results: .....

Special Diluent:

Manufacturer: .....

Catalogue no. manufacturer: .....

Batch number manufacturer: .....

Date(s) of preparation: .....

Batch no. laboratory: .....

Temperature of storage (°C): .....

pH (and measuring temp.): pH:..... measuring temperature:..... °C

Quality control results: .....

2.7 prEN 12780

Pseudomonas agar / CN-agar basal medium

Manufacturer: .....

Catalogue no. manufacturer: .....

Batch number manufacturer: .....

Date(s) of preparation: .....

Batch no. laboratory: .....

Temperature of storage (°C): .....

CN supplement:

Manufacturer: .....

Catalogue no. manufacturer: .....

Batch number manufacturer: .....

Date(s) of preparation: .....

Batch no. laboratory: .....

Temperature of storage (°C): .....

Complete medium:

pH (and measuring temp.): pH:..... measuring temperature:..... °C

Quality control results: .....

3. Give information on filters

Manufacturer: …….

Catalogue no.: .....

Batch number: …….

Expiry date: …….

Colour:…….

Diameter: ……. mm

Pore size: ……. µm

Give information on the quality control of the filters on a separate sheet, if necessary.

In case more than one type of filter is used, give the same information on the other type of filter.

REMARKS:

INCUBATION

4. How high were the Petri-dishes or microtiter plates stacked in the incubator?

Indicate number of Petri-dishes or microtiter plates in one stack

	Lenticules	Pastilles	Capsules
ISO 6222; 22 °C			
ISO 6222; 36 °C			
ISO/WD 6461-2			
ISO 7899-1 (MPN)			
ISO 7899-2 (mf)			
ISO 9308-1 (mf)			
ISO 9308-3 (MPN)			
prEN 12780			

5. Were the plates packed in plastic during incubation?

Indicate ‘yes’ (packed in plastic) or ‘no’ (not packed in plastic)

	Lenticules	Pastilles	Capsules
ISO 6222; 22 °C			
ISO 6222; 36 °C			
ISO/WD 6461-2			
ISO 7899-2 (mf)			
ISO 9308-1 (mf)			
prEN 12780			

6. What types of incubators were used?

22 °C:	☐ non fan-assisted	☐ fan-assisted
36 °C	☐ non fan-assisted	☐ fan-assisted
44 °C	☐ non fan-assisted	☐ fan-assisted

7. Temperature of refrigerators, freezers and incubators

If temperature of refrigerators, freezers and/or incubators are read automatically, please send a print-out of relevant period(s) of the relevant devices. Indicate which type of RM has been stored/incubated for which period and for which method in the relevant device.

If no automatic reading was carried out please:

- Give a list of min/max temperatures of the freezer ((-20 ± 5) °C) for the full period the RMs has been stored in it;
- Indicate for each method and type of RM the min/max temperatures, dates and times of the incubation in the tables below. Do not record low temperatures caused by opening the incubator. Therefore, place the thermometer in glycerol so that short and small temperature variations (e.g. caused by opening the door) do not lead to unnecessary actions.

Mind to use calibrated thermometers!

7.1 ISO 6222; (22 ± 2) °C for (68 ± 4) h

	Start incubation date; time	Finish incubation date; time	Temperature (°C)	
			min	max
Lenticules				
Pastilles				
Capsules				

7.2 ISO 6222; (36 ± 2) °C for (44 ± 4) h

	Start incubation date; time	Finish incubation date; time	Temperature (°C)	
			min	max
Lenticules				
Pastilles				
Capsules				

7.3 ISO/WD 6461-2; (44 ± 1) °C for (21 ± 3) h

	Start incubation date; time	Finish incubation date; time	Temperature (°C)	
			min	max
Lenticules				
Pastilles				
Capsules				

7.4 ISO 7899-1 (MPN); (44 ± 0.5) °C for 36-72 h

	Start incubation date; time	Finish incubation date; time	Temperature (°C)	
			min	max
Lenticules				
Pastilles				
Capsules				

7.5 ISO 7899-2 (mf); (36 ± 2) °C for (44 ± 4) h

	Start incubation date; time	Finish incubation date; time	Temperature (°C)	
			min	max
Lenticules				
Pastilles				
Capsules				

7.6 ISO 9308-1 (mf); (36 ± 2) °C for (21 ± 3) h

	Start incubation date; time	Finish incubation date; time	Temperature (°C)	
			min	max
Lenticules				
Pastilles				
Capsules				

7.7 ISO 9308-3 (MPN); (44 ± 0.5) °C for 36-72 h

	Start incubation date; time	Finish incubation date; time	Temperature (°C)	
			min	max
Lenticules				
Pastilles				
Capsules				

7.8 prEN 12780; (36 ± 2) °C for (44 ± 4) h

	Start incubation date; time	Finish incubation date; time	Temperature (°C)	
			min	max
Lenticules				
Pastilles				

REMARKS:

INFORMATION PER METHOD

8. ISO 6222: How did you control the temperature of the medium before pouring?

☐ In a control flask with a calibrated thermometer

☐ Label on the outside of the bottle

☐ Other, namely.....

9. ISO 6222: What was the temperature (in °C) of the medium just before pouring?

	Lenticules	Pastilles	Capsules
ISO 6222 – 22 °C	°C	°C	°C
ISO 6222 – 36 °C	°C	°C	°C

REMARKS:

INFORMATION PER TYPE OF REFERENCE MATERIAL

CAPSULES

10. According to SOP BCR-Water/001 the total reconstitution time of the capsules (time between addition of the first capsule to peptone-saline solution and placing the last tube in melting ice) should not exceed 50 minutes. Did it happen that this maximum time of 50 minutes was exceeded?

☐ no

☐ yes

If yes, please give information on the strain, batch and number of capsules and the relevant (ISO/EN-) method:

…….

…….

…….

11. Did you have problems with dissolution of the capsules?

☐ no

☐ yes

If yes, please give information on the strain, batch and number of capsules, kind of problems and the relevant (ISO/EN-) method:

…….

…….

…….

12. Did you have problems with membrane filtration of the capsule solutions (e.g. foaming, clogging of the filters)?

☐ no

☐ yes

If yes, please give information on the strain, batch and number of capsules, kind of problems and the relevant (ISO/EN-) method:

…….

…….

…….

REMARKS:

LENTICULES

13. Did you have problems with the use of lenticules?

☐ no

☐ yes

If yes, please give information on the strain, batch and number of lenticules, kind of problems and the relevant (ISO/EN-) method:

…….

…….

…….

REMARKS:

PASTILLES

14. Did you have problems with the use of pastilles?

☐ no

☐ yes

If yes, please give information on the strain, batch and number of pastilles, kind of problems and the relevant (ISO/EN-) method:

…….

…….

…….

REMARKS:

SIGNATURE

Name: ……

Date: ……

Signature: ……

Send this completed reporting form (on technical data) <u>before 5 July 2002</u> by e-mail, fax or by normal mail to:

Kirsten Mooijman
RIVM/MGB (Pb 63);
P.O.Box 1;
3720 BA Bilthoven ;
The Netherlands;
e-mail: kirsten.mooijman@rivm.nl
fax: +31 30 274 4434
(telephone: +31 30 274 3537)

Annex 9 Reporting form counts capsules

14-03-2002

REPORTING FORM

COUNT RESULTS OF CAPSULES
FEASIBILITY CERTIFICATION STUDIES OF MICROBIOLOGICAL REFERENCE MATERIALS

Laboratory name: .....

Labcode: .....

Contact person : .....

ISO 6222 (1999). Water quality – Enumeration of culturable micro-organisms – Colony count by inoculation in a nutrient agar culture medium

RM number	Count results (no. of cfp) after incubation at **22 °C**	
	count 1	count 2
1		
2		
3		
4		
5		

ISO 6222 (1999). Water quality – Enumeration of culturable micro-organisms – Colony count by inoculation in a nutrient agar culture medium

RM number	Count results (no. of cfp) after incubation at **36 °C**	
	count 1	count 2
1		
2		
3		
4		
5		

ISO/WD 6461-2 (2001). Water quality – Detection and enumeration of *Clostridium perfringens* – Part 2: Method by membrane filtration

RM number	Count results (no. of cfp)	
	count 1	count 2
1		
2		
3		
4		
5		

ISO 7899-1 (1998). Water quality – Detection and enumeration of intestinal enterococci in surface and waste water – Part 1: Miniaturized method (Most Probable Number) by inoculation in liquid medium

RM number	count 1		count 2	
	Number of positive wells 1/2 – 1/20	MPN/ 100 ml	Number of positive wells 1/2 – 1/20	MPN/ 100 ml
1	-		-	
2	-		-	
3	-		-	
4	-		-	
5	-		-	

ISO 7899-2 (2000). Water quality – Detection and enumeration of intestinal enterococci – Part 2: Membrane filtration method

RM number	Count results (no. of cfp)	
	count 1	count 2
1		
2		
3		
4		
5		

ISO 9308-1 (2000). Water quality – Detection and enumeration of *Escherichia coli* and coliform bacteria – Part 1: Membrane filtration method

RM number	Count results (no. of cfp)	
	count 1	count 2
1		
2		
3		
4		
5		

ISO 9308-3 (1998). Water quality – Detection and enumeration of *Escherichia coli* and coliform bacteria in surface and waste water – Part 3: Miniaturized method (Most Probable Number) by inoculation in liquid medium

RM number	count 1		count 2	
	Number of positive wells 1/2 – 1/20	MPN/ 100 ml	Number of positive wells 1/2 – 1/20	MPN/ 100 ml
1	-		-	
2	-		-	
3	-		-	
4	-		-	
5	-		-	

SIGNATURE

Name: ……

Date: ……

Signature: ……

DO NOT FORGET: Also complete the Excel file for capsule counts and send this file by e-mail to Kirsten Mooijman (RIVM/MGB-Bilthoven): kirsten.mooijman@rivm.nl

Send this completed reporting form (on counts of capsules) before 5 July 2002 by fax or by normal mail to:

Kirsten Mooijman
RIVM/MGB (Pb 63);
P.O.Box 1;
3720 BA Bilthoven ;
The Netherlands;
e-mail: kirsten.mooijman@rivm.nl
fax: +31 30 274 4434
(telephone: +31 30 274 3537)

Annex 10 Reporting form counts lenticules

14-03-2002

REPORTING FORM

COUNT RESULTS OF LENTICULES
FEASIBILITY CERTIFICATION STUDIES OF MICROBIOLOGICAL REFERENCE MATERIALS

Laboratory name: .....

Labcode: .....

Contact person : .....

ISO 6222 (1999). Water quality – Enumeration of culturable micro-organisms – Colony count by inoculation in a nutrient agar culture medium

RM number	Count results (no. of cfp) after incubation at **22 °C**	
	count 1	count 2
1		
2		
3		
4		
5		

ISO 6222 (1999). Water quality – Enumeration of culturable micro-organisms – Colony count by inoculation in a nutrient agar culture medium

RM number	Count results (no. of cfp) after incubation at **36 °C**	
	count 1	count 2
1		
2		
3		
4		
5		

ISO/WD 6461-2 (2001). Water quality – Detection and enumeration of *Clostridium perfringens* – Part 2: Method by membrane filtration

RM number	Count results (no. of cfp)
1	
2	
3	
4	
5	
6	
7	
8	
9	
10	

ISO 7899-1 (1998). Water quality – Detection and enumeration of intestinal enterococci in surface and waste water – Part 1: Miniaturized method (Most Probable Number) by inoculation in liquid medium

RM number	Number of positive wells 1/2 - 1/20	MPN/ 100 ml
1	-	
2	-	
3	-	
4	-	
5	-	
6	-	
7	-	
8	-	
9	-	
10	-	

ISO 7899-2 (2000). Water quality – Detection and enumeration of intestinal enterococci – Part 2: Membrane filtration method

RM number	Count results (no. of cfp)
1	
2	
3	
4	
5	
6	
7	
8	
9	
10	

ISO 9308-1 (2000). Water quality – Detection and enumeration of *Escherichia coli* and coliform bacteria – Part 1: Membrane filtration method

RM number	Count results (no. of cfp)
1	
2	
3	
4	
5	
6	
7	
8	
9	
10	

ISO 9308-3 (1998). Water quality – Detection and enumeration of *Escherichia coli* and coliform bacteria in surface and waste water – Part 3: Miniaturized method (Most Probable Number) by inoculation in liquid medium

RM number	Number of positive wells 1/2 - 1/20	MPN/ 100 ml
1	-	
2	-	
3	-	
4	-	
5	-	
6	-	
7	-	
8	-	
9	-	
10	-	

prEN 12780 (1999). Water quality – Detection and enumeration of *Pseudomonas aeruginosa* by membrane filtration

RM number	Count results (no. of cfp)
1	
2	
3	
4	
5	
6	
7	
8	
9	
10	

SIGNATURE

Name: ……

Date: ……

Signature: ……

DO NOT FORGET: Also complete the Excel file for lenticule counts and send this file by e-mail to Dave Stewardson (University-Newcastle): D.J.Stewardson@ncl.ac.uk

Send this completed reporting form (on counts of lenticules) before 5 July 2002 by fax or by normal mail to:

Dave Stewardson
ISRU, MMME
University of Newcastle Upon Tyne
Stephenson Building
Claremont road
NE1 7RU
Newcastle upon Tyne
England
fax: +44 191 261 2578
(telephone: +44 191 261 2577)

Annex 11 Reporting form counts pastilles

15-03-2002

REPORTING FORM

COUNT RESULTS OF PASTILLES
FEASIBILITY CERTIFICATION STUDIES OF MICROBIOLOGICAL REFERENCE MATERIALS

Laboratory name: .....

Labcode: .....

Contact person : .....

ISO 6222 (1999). Water quality – Enumeration of culturable micro-organisms – Colony count by inoculation in a nutrient agar culture medium

RM number	Count results (no. of cfp) after incubation at **22 °C**	
	count 1	count 2
1		
2		
3		
4		
5		

ISO 6222 (1999). Water quality – Enumeration of culturable micro-organisms – Colony count by inoculation in a nutrient agar culture medium

RM number	Count results (no. of cfp) after incubation at **36 °C**	
	count 1	count 2
1		
2		
3		
4		
5		

ISO/WD 6461-2 (2001). Water quality – Detection and enumeration of *Clostridium perfringens* – Part 2: Method by membrane filtration

RM number	Count results (no. of cfp)
1	
2	
3	
4	
5	
6	
7	
8	
9	
10	

ISO 7899-1 (1998). Water quality – Detection and enumeration of intestinal enterococci in surface and waste water – Part 1: Miniaturized method (Most Probable Number) by inoculation in liquid medium

RM number	Number of positive wells 1/2 - 1/20	MPN/ 100 ml
1	-	
2	-	
3	-	
4	-	
5	-	
6	-	
7	-	
8	-	
9	-	
10	-	

ISO 7899-2 (2000). Water quality – Detection and enumeration of intestinal enterococci – Part 2: Membrane filtration method

RM number	Count results (no. of cfp)
1	
2	
3	
4	
5	
6	
7	
8	
9	
10	

ISO 9308-1 (2000). Water quality – Detection and enumeration of *Escherichia coli* and coliform bacteria – Part 1: Membrane filtration method

RM number	Count results (no. of cfp)
1	
2	
3	
4	
5	
6	
7	
8	
9	
10	

ISO 9308-3 (1998). Water quality – Detection and enumeration of *Escherichia coli* and coliform bacteria in surface and waste water – Part 3: Miniaturized method (Most Probable Number) by inoculation in liquid medium

RM number	Number of positive wells 1/2 - 1/20	MPN/ 100 ml
1	-	
2	-	
3	-	
4	-	
5	-	
6	-	
7	-	
8	-	
9	-	
10	-	

prEN 12780 (1999). Water quality – Detection and enumeration of *Pseudomonas aeruginosa* by membrane filtration

RM number	Count results (no. of cfp)
1	
2	
3	
4	
5	
6	
7	
8	
9	
10	

SIGNATURE

Name: ……

Date: ……

Signature: ……

DO NOT FORGET: Also complete the Excel file for pastille counts and send this file by e-mail to Tristan Simonart (Inst. Pasteur Lille): tristan.simonart@pasteur-lille.fr

Send this completed reporting form (on counts of pastilles) before 5 July 2002 by fax or by normal mail to:

Tristan Simonart;
Institute Pasteur of Lille;
Water and Environment Department;
1, Rue du Professeur Calmette
P.O.Box 245
59019 Lille
France
fax: +33 3 20 87 73 83
(telephone: +33 33 20 87 72 60)

Annex 12 Technical results

21 February 2003

MICROCRM
FEASIBILITY CERTIFICATION STUDIES OF MICROBIOLOGICAL REFERENCE MATERIALS
Technical results

…….. : Deviating from protocol; might have had influence on the results

A.12.1 General

Labcode	Arrival parcel in the laboratory[1]			General remarks
	Lenticules	Pastilles	Capsules	
1	270302	260302	270302	
2	270302	260302	270302	
3	260302	260302	020402	
4	260302	260302	no transport	Most of the pastille RMs had orange silicagel before opening the vials
5	260302	260302	270302	
6	270302	260302	280302	
7	270302	260302	020402	It was difficult to take the pastilles out of the containing vials
8	020402	290302	050402	Waterbath for reconstitution of the capsules was controlled at (38 ± 1) °C instead of (38 ± 0.5) °C
9	280302	280302	090402	
10	260302	260302	270302	Did not use the filters of IPL as the colonies were difficult to count (used own filters)
11	260302	260302	270302	
12	no transport	260302	260302	
13	270302	no transport	270302	

[1]: Date of mailing 25/26 March 2002

Other general technical info

- Storage of the RMs at (-20 ± 5) °C: Lab 1 and 9 reported incidental temperatures warmer than –15 °C (lab 1 up to 0 °C and lab 9 up to –12 °C), but they indicated that these temperatures existed only for short periods (ca < 30 min). Lab 2 reported periods of very cold temperatures (up to –32 °C). It is not expected that temperatures colder than –25 °C has had any influence on the RMs. Of the other laboratories the temperature of the freezer was within the prescribed limits during the period the RMs were stored.
- Filters: Ten laboratories (labcodes 1, 2, 3, 5, 6, 8, 9, 10, 12, 13) used Millipore filters (most of them supplied by IPL). Two laboratories (labcodes 4, 11) used Sartorius filters. One laboratory (labcode 7) used Schleicher and Schuell filters.
- Transport time: If transport time was more than 3 days the temperatures and times during transport were as follows:
  - Lab 8 lenticules: most of the time at ca 4 °C
  - Lab 8 pastilles: most of the time at ≤ 0 °C
  - Lab 3 capsules: < 10 °C: ca 84 h; 10-15 °C ca 66 h; 15-20 °C: ca 24 h; 20-25 °C: ca 1 h;
  - Lab 7 capsules: < 10 °C: ca 120 h; 10-15 °C: ca 12 h; 15-20 °C: ca 30 h; 20-25 °C: ca 10 h.
  - Lab 8 capsules: ca 1,5 day at 15 °C, rest of the time at < 10 °C (varying from –20 °C to +9 °C)
  - Lab 9 capsules: ca 15 days at ca 15 °C.

Conclusions general technical info

Of the general technical information the critical point has been the transport time. Long transport time at elevated temperatures (ca 15 °C), might have had influence on the results of the less stable RMs like *Escherichia coli* and *Pseudomonas aeruginosa*.

It is not necessary to exclude the data of laboratories with long transport time on forehand, but it would be advisable to screen the data of these laboratories to find out whether they found relative low results. Check data of lab 8 (all RMs), 3 (capsules), 7 (capsules) and 9 (capsules).

A.12.2 ISO 6222; Colony count with incubation at 22 °C and at 36 °C

Labcode	Date of analyses [1] lent.; past.; caps.	Pour temperature of YA (°C)[2]	Way of measuring pour temperature	No. of Petri dishes at max in one stack[3]	Plates packed in plastic?
1	06/05/02	45	control flask	4	no
2	21/05; 14/05; 03/06	41 – 42	label outside	2	no
3	08/05/02	43 – 44	label outside	5	yes
4	14/05/02	45.5	control flask	3	yes
5	14/05; 14/05; 13/05	45	control flask & label outside	4	yes
6	24/06/02	44	control flask & label outside	4	yes
7	27/05/02	45	label outside	10	no
8	27/06 (22 °C and caps 36 °C); 28/06	43 – 44	label outside	10	no
9	21/05/02	42	label outside	2	no
10	28/06/02	44 – 45	label outside	10 (22) / 4 (36)	no (22) / yes (36)
11	29/05/02	43.6	control flask	5	no
12	15/07/02	44	control flask	5	no
13	05/07/02	42 – 47	label outside	5	no, closed boxes

According to protocol/SOPs:
[1]: 1 April – 30 June
[2]: (45 ± 1) °C
[3]: Not prescribed. ISO/CD 8199 (2002) indicates: ‘Do not make higher stacks than 6 Petri dishes’.

Other technical info concerning ISO 6222

- Yeast Extract agar (YA):
  - In 8 laboratories (labcodes 1, 2, 3, 5, 7, 8, 10, 13) the manufacturer was Biorad (mostly supplied by IPL). One laboratory (labcode 6) used Lab M. Two laboratories (labcode 9, 12) used Oxoid. One laboratory (labcode 11) used Merck. One laboratory (labcodes 4) used 'house made' YA.
  - pH of YA was of all laboratories within the prescribed range (7.2 ± 0.2 at 25 °C).
- Incubation:
  - Incubation time and temperature range of the incubation at 22 °C was for all laboratories within the prescribed limits [(22 ± 2) °C for (68 ± 4) h)].
  - Incubation time and temperature range of the incubation at 36 °C was for all laboratories within the prescribed limits [(36 ± 2) °C for (44 ± 4) h)].
- General remarks:
  - Lab 6 found very low counts (10-fold lower than expected) for capsules at 22 °C and at 36 °C. No explanation was found.
  - Lab 7 did not analyse duplicates for lenticules nor for pastilles (but analysed 10 in singular instead of 5 in duplicate).

Conclusions concerning ISO 6222

- Date of analyses later than prescribed: no real influence on results expected.
- Pour temperature of YA deviating from prescribed temperature: too low – no influence on results expected; too high – might result **in lower** counts. **Check data of lab 13.**
- High stacks of Petri dishes: can cause an uneven distribution of the incubation temperature in the plates and might therefore cause somewhat higher variation in results. **Check data of lab 7 (22 °C and 36 °C), 8 (22 °C and 36 °C) and 10 (22 °C).**
- **Lab 7 did not analyse duplicates for lenticules nor for pastilles. Delete data for further analyses.**

A.12.3 ISO/WD 6461-2 (May 2001); Clostridium perfringens

Lab-code	Date of analyses [1] lent.; past.; caps.	pH of TSC[2] (measuring temp. °C)	Temp. incubator min/max (°C)[3]	Incubation time (h)[4] lent.; past.; caps.	Remarks
1	07/05/02	7.3 (50)	43.0 / 44.8	22	QC: no growth on TSC; Lenticules: many white colonies
2	22/05; 15/05; 03/06	7.4	43.4 / 44.3	46.5 ; 26 ; 22.5	No typical colonies (all RMs)
3	23/04/02	7.5 (22)	43.2 / 44.4	24	QC: growth only after 72 h; Lenticules: white colonies after 24 h, black after 40 h
4	16/04/02	7.1 (45)	43.3 / 44.4	24.5	Lenticules: white colonies
5	16/05; 16/05; 21/05	7.4 (25)	43.9 / 44.2	24 ; 24 ; 20.5	
6	28/06/02	7.45 (25)	44.1 / 44.2	24	
7	03/06/02	7.4 (20)	44.0	42.5 ; 43 ; 44	Lenticules: nearly invisible colonies; Capsules: solution not shaken before mf
8	27/06/02	7.3 (25)	43.7 /45.1	24	QC: no growth, but no problems with tests
9	28/06/02	7.8 (36)	37.0 / 37.2	23	
10	02/07/02	7.4 (24)	43.8 / 44.0	22 ; 23.5 ; 22	QC: no good results, used own TSC; Lenticules: colonies not black Capsules: some colonies yellow not black
11	11/06/02	7.3 (25)	43.0 / 44.3	21	QC: after 24h no growth, after 48 h : OK; Lent. and caps.: no or few typical colonies
12	15/07/02	7.7	44.0 / 44.1	24	Overlay of TSC over membrane
13	04/07/02	7.4 (20)	43.8 / 44.3	41.5 ; 40.5 ; 41.5	Many non-typical colonies for all RMs

According to protocol/SOPs:
[1]: 1 April – 30 June; [2]: TSC: Tryptose Sulphite Cycloserine agar, pH 7.6 ± 0.2 (no temperature indication); [3]: (44 ± 1) °C; [4]: (21 ± 3) h

Other technical info concerning ISO/WD 6461-2 (May 2001)

- Tryptose Sulphite Cycloserine agar without egg yolk (TSC):
  In 8 laboratories (labcodes 1, 2, 3, 5, 7, 8, 11, 13) the manufacturer was Scharlau (mostly supplied by IPL). One laboratory (labcode 4) used Merck. One laboratory (labcode 6) used Difco. One laboratory (labcode 9) used Oxoid. One laboratory (labcode 10) used Biocar. One laboratory (labcode 12) used 'house made' TSC.

Conclusions concerning ISO/WD 6461-2 (May 2001)

- Date of analyses later than prescribed: no real influence on results expected.
- Deviating pH of TSC: influence unknown. **Check data of lab 4, 8, 11 (also for missing info lab 10).**
- **Incubation temperature of lab 9 was 36 °C instead of 44 °C. Delete data for further analyses.**
- **Incubation time of lab 2 (lenticules), 7 (all RMs), 13 (all RMs) was ca 48 h instead of 24 h. Delete data for further analyses.**
- Lab 7 did not shake the capsule solution before filtration. This might result in somewhat deviating results (either low or high or more variation). **Check the results of lab 7 (capsules).**
- According to ISO/ WD 6461-2 the colonies to be counted should 'show a blackening however faint of the TSC medium when viewed from either above or below the membrane filter'. 'Colonies are usually black to yellow brown'. **Following this 'rule' the following data should be deleted for further analyses:**
  - **Lab 1 lenticules;**
  - **Lab 2 lenticules, pastilles and capsules;**
  - **Lab 3 lenticules;**
  - **Lab 4 lenticules;**
  - **Lab 7 lenticules;**
  - **Lab 10 lenticules and capsules;**
  - **Lab 11 lenticules and capsules;**
  - **Lab 13 lenticules, pastilles (some results) and capsules.**

A.12.4 ISO 7899-1; Intestinal Enterococci miniaturised MPN

Labcode	Date of analyses [1] lent.; past.; caps.	No. of plates at max in one stack[2] lent.; past.; caps.	Temp. incubator min/max (°C)[3]	Incubation time (h)[4] lent.; past.; caps.
1	21/05/02	5	43.0 / 44.6	47 ; 46.5 ; 47
2	21/05 ; 14/05 ; 04/06	5	43.4 / 44.3	70.5 ; 70 ; 70.5
3	30/04/02	10	43.6 / 44.2	66.5 ; 67.5 ; 63.5
4	22/05/02	3	43.4 / 44.1	44
5	21/06 ; 20/06 ; 24/06	4	43.9 / 44.4	72 ; 52 ; 65
6	03/06 ; 04/06 ; 03/06	5	44.0 / 44.2	46.5 ; 47 ; 46.5
7	27/05/02	5	44.0	43.5 ; 44.5 ; 41
8	19/06 ; 19/06 ; 28/06	10	44.0 / 44.9	48 ; 48 ; 46
9	11/06/02	3	44	68.5
10	03/07/02	10	43.8 / 44.2	43.5 ; 44.5 ; 41.5
11	18/06/02	2	42.3 / 45.3*	43.5
12	15/07/02	5	44.0 / 44.2	46
13	02/07 ; 03/07 ; 02/07	5	43.4 / 44.2	45.5 ; 45 ; 45.5

According to protocol/SOPs:
[1]: 1 April – 30 June;
[2]: Not prescribed. ISO/CD 8199 (2002) indicates: 'Do not make higher stacks than 6 Petri dishes'. Also sound for microtiter plates?
[3]: (44 ± 0.5) °C;
[4]: 36 – 72 h
*: Short period of exceeding temperature limits

Other technical info concerning ISO 7899-1

- MUD/SF medium (microtiter plates):
  All laboratories used plates of Biorad (most of them supplied by IPL).
- Special diluent (SD):
  All laboratories used SD of Biorad (most of them supplied by IPL), except laboratory 13 who used SD of Aquasystems. Eleven laboratories reported a pH of the SD of 7.0-7.2. Laboratory 2 and 13 reported a pH of 7.4.

Conclusions concerning ISO 7899-1

- Date of analyses later than prescribed: no real influence on results expected.
- Incubation temperatures deviating from prescribed temperatures. The deviations are only small or short: no real influence on results expected.
- High stacks of microtiter plates: can cause an uneven distribution of the incubation temperature in the plates and might therefore cause somewhat higher variation in results. **Check data of lab 3, 8, 10.**
- The pH of SD was not prescribed. No problems in the range of 7.0-7.4 are expected.

A.12.5 ISO 7899-2; Intestinal Enterococci membrane filtration

Labcode	Date of analyses [1] lent.; past.; caps.	pH of S&B[2] (measuring temp. °C)	No. of Petri dishes at max in one stack[3] lent.; past.; caps.	Plates packed in plastic?	Temp. incubator min/max (°C)[4]	Incubation time (h)[5] lent.; past.; caps.
1	27/05/02	7.2 (45)	4	no	35.3 / 37.5	45.5; 46.5; 45
2	22/05; 15/05; 29/05	7.2	5	no	36.6 / 37.3	47.5;49;45.5
3	24/04/02	7.1 (20)	5	yes	34.1 / 36.9	46
4	10/04/02	within range	3	yes	36.5 / 37.2	45
5	19/06; 19/06; 20/06	7.2-7.3 (25)	4	yes	35.7 / 35.8	48
6	18/06/02	7.2 (25)	3	yes	36.9 / 37.0	46
7	03/06/02	7.2 (20)	10	no	36.0 / 36.8	45.5; 45.5; 46.5
8	24/06; 24/06; 27/06	7.2 (25)	10	no	35.8 / 37.3	48
9	24/05/02	7.4 (40)	1	yes	36.9 / 37.0	48
10	05/06; 05/06; 06/06	7.4 (22)	4	yes	36.4 / 37.3	44
11	13/06/02	7.2 (25)	5	no	35.8 / 36.4	46
12	15/07/02	7.3	5	no	36.6 / 37.0	48
13	04/07; 04/07; 03/07	7.4 (20)	5	no	36.3 / 37.8	40.5; 40.5; 45.5

According to protocol/SOPs:
[1]: 1 April – 30 June;
[2]: S&B: Slanetz and Bartley, pH 7.2 ± 0.1 at 25 °C
[3]: Not prescribed. ISO/CD 8199 (2002) indicates: 'Do not make higher stacks than 6 Petri dishes'.
[4]: (36 ± 2) °C
[5]: (44 ± 4) h

Other technical info concerning ISO 7899-2

- Slanetz and Bartley (S&B) medium basal:
  In seven laboratories (labcodes 1, 2, 3, 5, 7, 8, 10) the manufacturer was Biorad (mostly supplied by IPL). One laboratory (labcode 4) used Merck. Two laboratories (labcodes 6, 11) used Oxoid. One laboratory (labcode 9) used Lab M. One laboratory (labcode 13) used Sanofi. One laboratory (labcode 12) used 'house made' S&B.
- TTC:
  In eight laboratories (labcodes 1, 2, 3, 5, 7, 8, 10, 13) the manufacturer was Merck (mostly supplied by IPL). Five laboratories (labcodes 4, 6, 9, 11, 12) gave no info about the manufacturer of TTC. This could also mean that TTC was already included in the 'basal' medium.
- Remarks:
  - Lab 1 reported no growth of the capsules on S&B
  - Lab 7 reported that the capsules solution was not shaken before membrane filtration

Conclusions concerning ISO 7899-2

- Date of analyses later than prescribed: no real influence on results expected.
- Deviating pH of S&B: influence unknown. However, the deviations are only small: no real influence on results expected.
- High stacks of Petri dishes: can cause an uneven distribution of the incubation temperature in the plates and might therefore cause somewhat higher variation in results. **Check data of lab 7, 8.**
- Lab 1 did not found growth of the capsules on S&B. An unknown technical error might have caused this. The lab preferred to **enter the results of lab 1 (capsules) as missing.**
- Lab 7 did not shake the capsule solution before filtration. This might result in somewhat deviating results (either low or high or more variation). **Check the results of lab 7 (capsules).**

A.12.6 ISO 9308-1; E. coli and coliforms membrane filtration

Labcode	Date of analyses [1] lent.; past.; caps.	pH of LTTC[2] (measuring temp. °C)	No. of Petri dishes at max in one stack[3] lent.; past.; caps.	Plates packed in plastic?	Temp. incubator min/max (°C)[4]	Incubation time (h)[5] lent.; past.; caps.
1	10/05/02	7.1 (45)	4	no	35.3 / 37.5	21.5
2	22/05; 15/05; 29/05	7.2	5	no	36.6 / 37.3	25; 24; 23
3	07/05/02	7.3 (22)	10	no	35.2 / 36.0	23
4	11/04/02	within range	3	no	36.5 / 37.2	22
5	06/06; 06/06; 07/06	7.3 (25)	4	yes	35.8 / 35.9	24; 22.5; 24.5
6	12/06; 12/06; 13/06	7.3 (25)	3	yes	36.8 / 37.1	22; 22; 22.5
7	28/05/02	7.2 (20)	10	no	36.0 / 36.1	23; 22.5; 22.5
8	24/06; 24/06; 28/06	7.1 (24)	10	no	35.8 / 37.4	24
9	06/06/02	LSA	2	yes	37.0 / 37.2	22
10	22/05/02	7.4 (22)	4	yes	36.4 / 37.1	23; 23; 21
11	06/06/02	7.0 (25)	5	no	35.4 / 36.2	22
12	16/07/02	7.1	5	no	36.4 / 37.0	24
13	03/07; 04/07; 03/07	7.4 (22)	5	no	36.3 / 37.6	46; 40.5; 46

According to protocol/SOPs:
[1]: 1 April – 30 June;
[2]: LTTC: Lactose TTC agar with Tergitol-7, pH 7.2 ± 0.1 at 25 °C
[3]: Not prescribed. ISO/CD 8199 (2002) indicates: ‘Do not make higher stacks than 6 Petri dishes’.
[4]: (36 ± 2) °C
[5]: (21 ± 3) h

Other technical info concerning ISO 9308-1

- Lactose TTC agar with Tergitol-7 (LTTC) medium basal:
  In seven laboratories (labcodes 1, 2, 3, 5, 7, 8, 10) the manufacturer was Biorad (mostly supplied by IPL). Two laboratories (labcodes 4, 11) used Oxoid. Two laboratories (labcodes 6, 13) used Sanofi. One laboratory (labcode 13) used Sanofi. One laboratory (labcode 12) used Merck (basal medium including Tergitol 7). One laboratory (labcode 9) used Lauryl Sulphate agar (LSA) instead of LTTC.
- TTC:
  In seven laboratories (labcodes 2, 3, 5, 7, 8, 10, 13) the manufacturer was Biorad (mostly supplied by IPL). Two laboratories (labcodes 1, 11) used Merck. One laboratory (labcode 6) used Oxoid. One laboratory (labcode 12) used Sigma. One laboratory (labcode 4) gave no information about the manufacturer of TTC. This could also mean that TTC was already included in the 'basal' medium. No information concerning TTC of Lab 9 as they used a different medium.
- Tergitol-7:
  In eight laboratories (labcodes 1, 2, 3, 5, 7, 8, 10, 13) the manufacturer was Biorad (mostly supplied by IPL). One laboratory (labcode 6) used Sigma. Three laboratories (labcodes 4, 11, 12) gave no information about the manufacturer of Tergitol-7. This could also mean that Tergitol-7 was already included in the 'basal' medium. No information concerning Tergitol-7 of Lab 9 as they used a different medium.
- According to ISO 9308-1 complete poured plates of LTTC can be stored at (5 ± 3) °C, but should be used within 10 days. Ten laboratories used their complete plates within 10 days after preparation. One laboratory (labcode 9) did no use LTTC. Two laboratories (labcodes 4, 10) used their plates after respectively 12 days and 15 days of storage. However, a small test with capsule RMs, performed in lab 4 had shown that the mean level of the 9 capsules tested in duplicate of one batch of *E.coli* did not differ when tested on LTTC of an age of 2 days (mean: 58 cfp) and on LTTC with an age of one month (mean: 63 cfp).
- Remarks:
  - Lab 11 reported for lenticules one plate with 'blurred' (non-readable) colonies.
  - Lab 13 reported for lenticules 2 types of colonies on all plates. Both types were confirmed with API 20E as *E. coli*.

Conclusions concerning ISO 9308-1

- Date of analyses later than prescribed: no real influence on results expected.
- Deviating pH of LTTC: influence unknown. However, the deviations are only small: no real influence on results expected.
- High stacks of Petri dishes: can cause an uneven distribution of the incubation temperature in the plates and might therefore cause somewhat higher variation in results. **Check data of lab 3, 7, 8.**
- **Incubation time of lab 13 was ca 44 h instead of ca 21 h. Delete data for further analyses.**
- **Lab 9 used LSA instead of LTTC. Delete data for further analyses.**
- Lab 4 and 10 used LTTC plates, which had been stored for more than 10 days (12 and 15 days). However, taking into account the test of lab 4: no real influence on results is expected.
- Lab 11 reported for lenticules one plate with ‘blurred’ (non-readable) colonies. **Enter a missing result for lab 11 lenticules unit 6.**
- Lab 13 reported for lenticules 2 types of colonies on all plates, both types were confirmed as *E. coli*. **Enter the total number of both types of colonies for lab 13, lenticules.**

A.12.7 ISO 9308-3; E. coli miniaturised MPN

Labcode	Date of analyses [1] lent.; past.; caps.	No. of plates at max in one stack[2] lent.; past.; caps.	Temp. incubator min/max (°C)[3]	Incubation time (h)[4] lent.; past.; caps.
1	15/05/02	5	43.2 / 44.9	43.5 ; 44.5 ; 43
2	21/05 ; 14/05 ; 04/06	5	43.4 / 44.3	70 ; 71 ; 71
3	06/05/02	10	43.7 / 44.2	66 ; 66 ; 62.5
4	05/07/02	3	43.4 / 44.1	47
5	23/06 ; 21/06 ; 24/06	4	43.9 / 44.4	68 ; 71 ; 66
6	25/06 ; 19/06 ; 17/06	5	44.0 / 44.3	46 ; 47 ; 47
7	27/05/02	5	43.4 / 44.2	43.5 ; 44.5 ; 42
8	20/06 ; 20/06 ; 28/06	10	43.8 / 44.7	48 ; 49 ; 48
9	12/06/02	3	44	45.5 ; 45.5 ; 44.5
10	03/07/02	10	43.8 / 44.2	48 ; 48 ; 41.5
11	05/06; 05/06; 04/06	2	43.5 / 45.2	41 ; 41 ; 48
12	16/07/02	5	43.5 / 44.1	46
13	02/07 ; 03/07 ; 02/07	5	43.4 / 44.2	45.5 ; 45 ; 45.5

According to protocol/SOPs:
[1]: 1 April – 30 June;
[2]: Not prescribed. ISO/CD 8199 (2002) indicates: ‘Do not make higher stacks than 6 Petri dishes’. Also sound for microtiter plates?
[3]: (44 ± 0.5) °C;
[4]: 36 – 72 h

Other technical info concerning ISO 9308-3

- MUG/EC medium (microtiter plates):
  All laboratories used plates of Biorad (most of them supplied by IPL).
- Special diluent (SD):
  All laboratories used SD of Biorad (most of them supplied by IPL), except laboratory 13 who used SD of Aquasystems. Eleven laboratories reported a pH of the SD of 7.0-7.2. Laboratory 2 and 13 reported a pH of 7.4.

Conclusions concerning ISO 9308-3

- Date of analyses later than prescribed: no real influence on results expected.
- Incubation temperatures deviating from prescribed temperatures. The deviations are only small or short: no real influence on results expected.
- High stacks of microtiter plates: can cause an uneven distribution of the incubation temperature in the plates and might therefore cause somewhat higher variation in results. **Check data of lab 3, 8, 10.**
- The pH of SD was not prescribed. No problems in the range of 7.0-7.4 are expected.

A.12.8 prEN 12780: Pseudomonas aeruginosa membrane filtration

Labcode	Date of analyses [1] lent.; past.	No. of Petri dishes at max in one stack[2] lent.; past.	Plates packed in plastic?	Temp. incubator min/max (°C)[3]	Incubation time (h)[4] lent.; past.
1	29/05/02	4	no	35.3 / 37.5	48
2	22/05 ; 15/05	5	no	36.6 / 37.3	48 ; 46.5
3	09/05/02	10	yes	34.1 / 36.2	48
4	17/04/02	3	yes	35.8 / 37.1	46
5	21/05/02	4	yes	35.8 / 35.9	43.5 ; 45.5
6	25/04/02	3	yes	36.8 / 37.1	47
7	04/06/02	10	yes	36.2 / 37.0	45 ; 44
8	25/06/02	10	no	35.4 / 37.3	48 ; 47
9	21/05/02	2	yes	36.6 / 37.2	43
10	29/05/02	4	yes	36.4 / 37.3	45
11	10/06/02	5	no	35.5 / 36.3	47.5
12	15/07/02	5	no	36.1 / 37.0	45.5
13	08/07/02	5	no	35.8 / 37.9	47.5

According to protocol/SOPs:
[1]: 1 April – 30 June;
[2]: Not prescribed. ISO/CD 8199 (2002) indicates: 'Do not make higher stacks than 6 Petri dishes'.
[3]: (36 ± 2) °C;
[4]: (44 ± 4) h (examine the plates for growth after (21 ± 3) h and after (44 ± 4) h).

Other technical info concerning prEN 12780

- CN-agar base:
  In eleven laboratories (labcodes 1, 2, 3, 4, 5, 7, 8, 9, 10, 11, 13) the manufacturer was Oxoid (mostly supplied by IPL). One laboratory (labcode 6) used Lab M. One laboratory (labcode 12) used 'house made' CN-agar.
- CN-supplement:
  In twelve laboratories (labcodes 1, 2, 3, 4, 5, 7, 8, 9, 10, 11, 12, 13) the manufacturer was Oxoid (mostly supplied by IPL). One laboratory (labcode 6) used Lab M.
- Of all laboratories the pH of the complete medium was within the prescribed range of 7.1 ± 0.2.
- Remarks:
  Lab 8 reported that the colonies on the membranes were difficult to count. The colonies were big and 'smearing' and difficult to differentiate. Counts were indicated as 'approximates'.

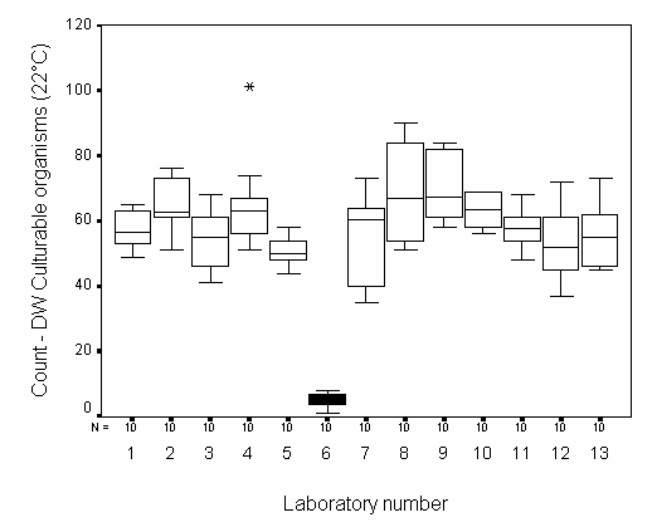
Conclusions concerning prEN 12780

- Date of analyses later than prescribed: no real influence on results expected.
- High stacks of Petri dishes: can cause an uneven distribution of the incubation temperature in the plates and might therefore cause somewhat higher variation in results. **Check data of lab 3, 7, 8.**
- Lab 8 had difficulties with reading of the plates. Because colonies were big and 'smearing' more than 2 colonies might have been counted as one, resulting in a low mean count. **Check data of lab 8.**

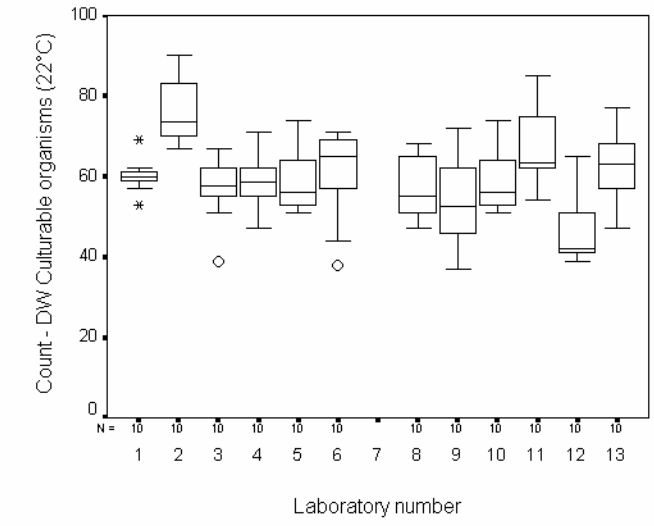
Annex 13 Box and Whisker plots

ISO 6222 Culturable organisms at 22 °C

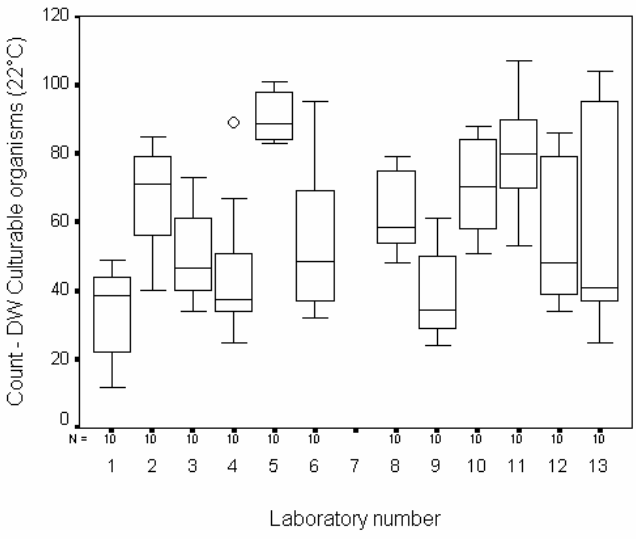
Capsules



Lenticules

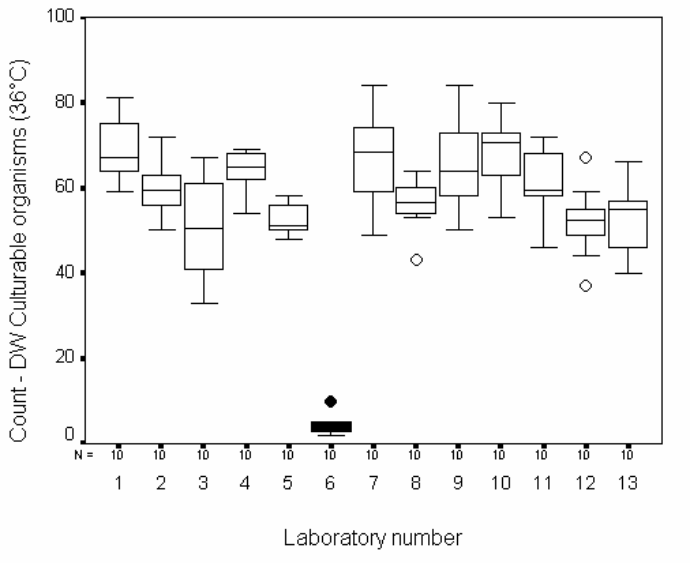


Pastilles

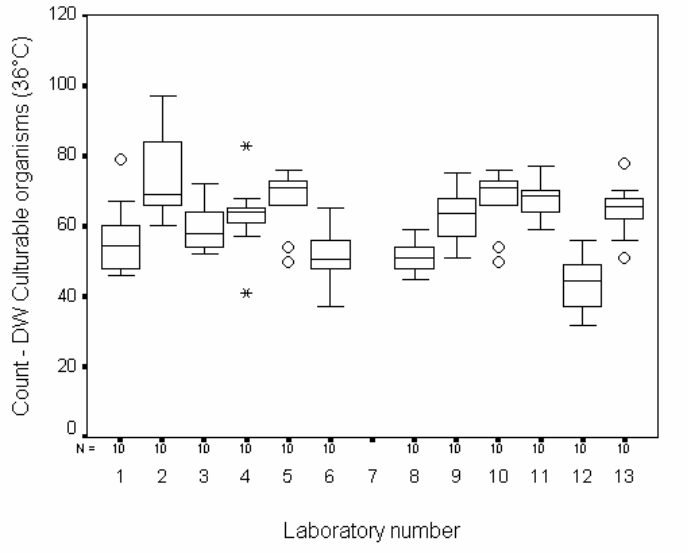


ISO 6222 Culturable organisms at 36 °C

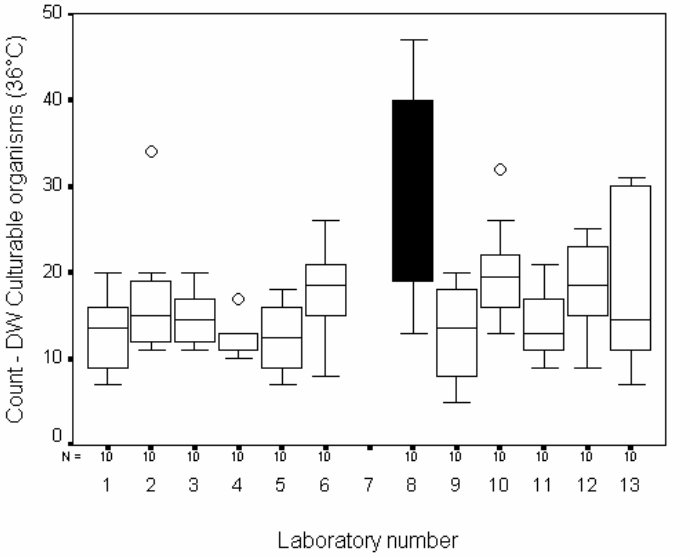
Capsules



Lenticules

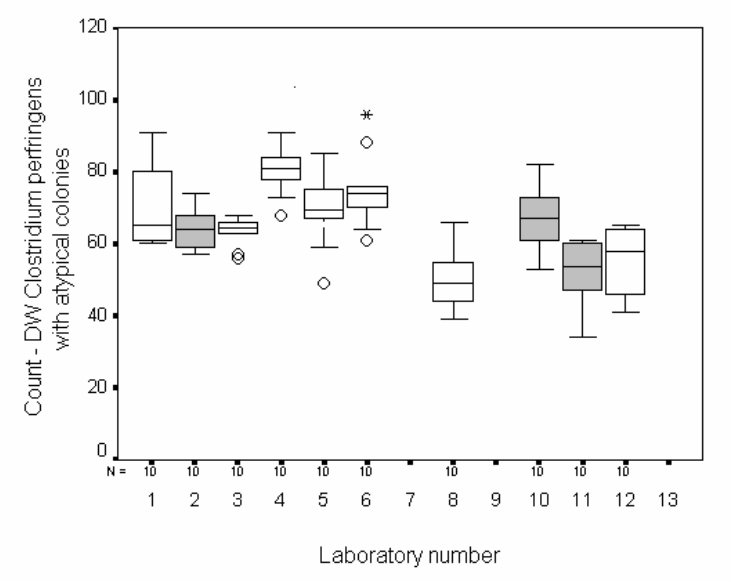


Pastilles

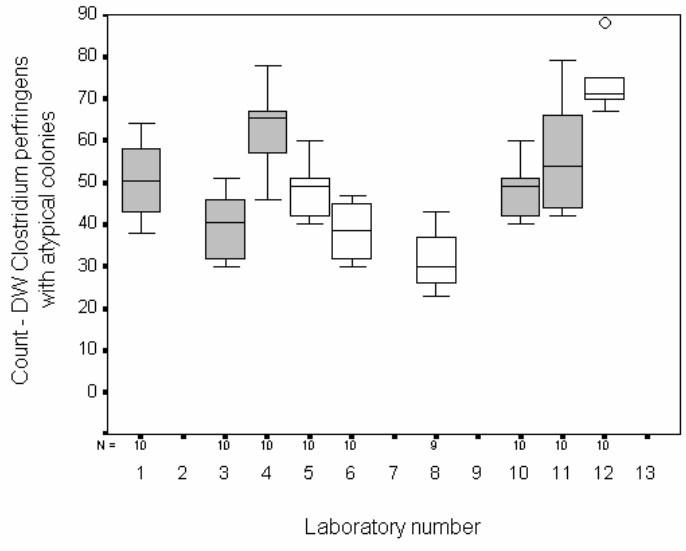


ISO/WD 6461-2 *Clostridium perfringens*: Results of typical and atypical colonies (grey)

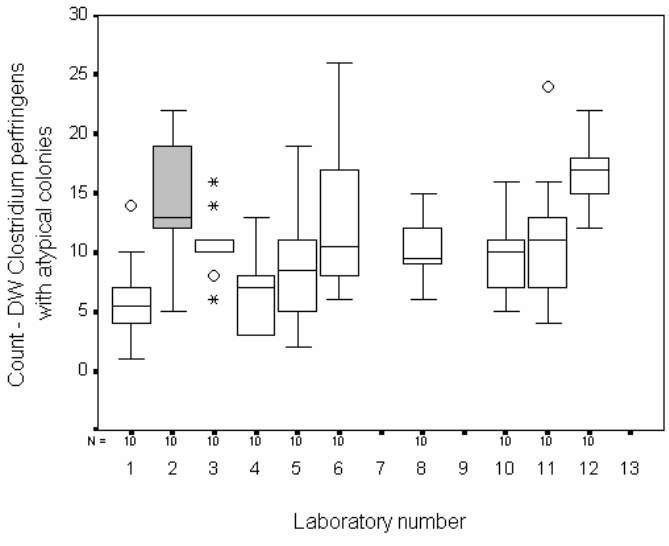
Capsules



Lenticules

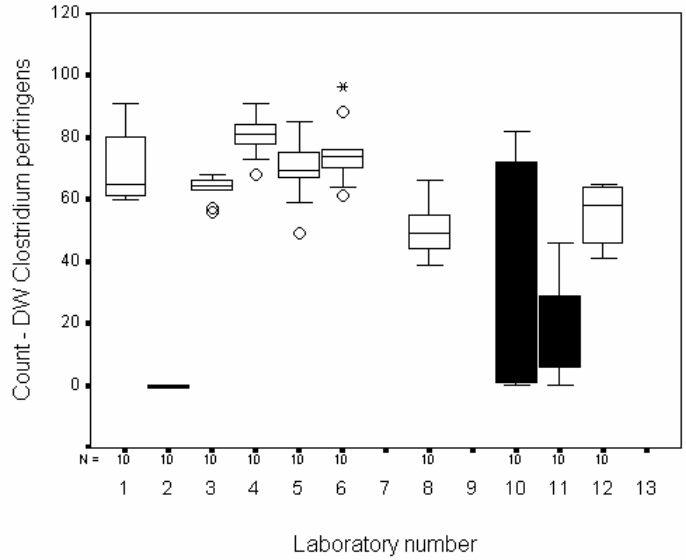


Pastilles

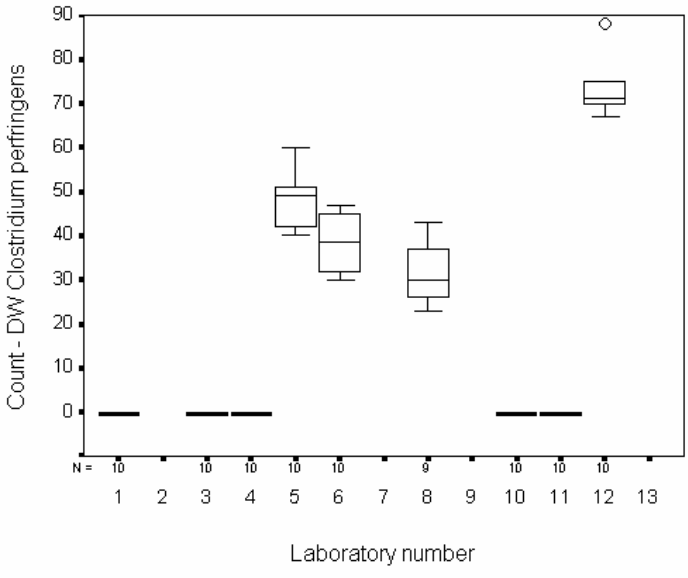


ISO/WD 6461-2 *Clostridium perfringens*: Results of (only) typical colonies

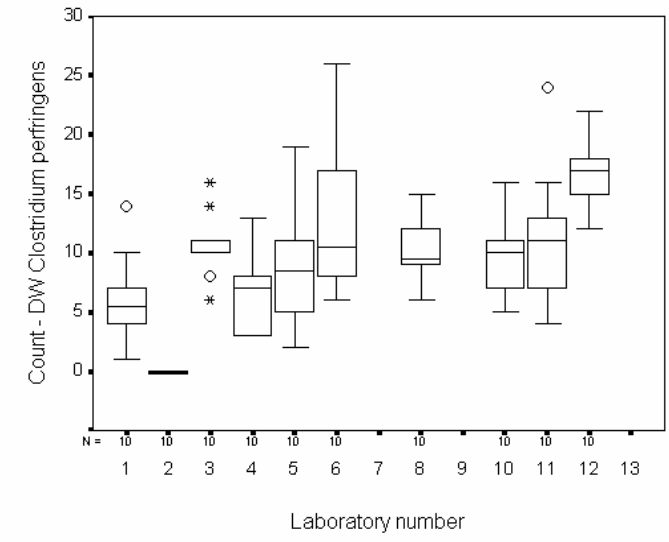
Capsules



Lenticules

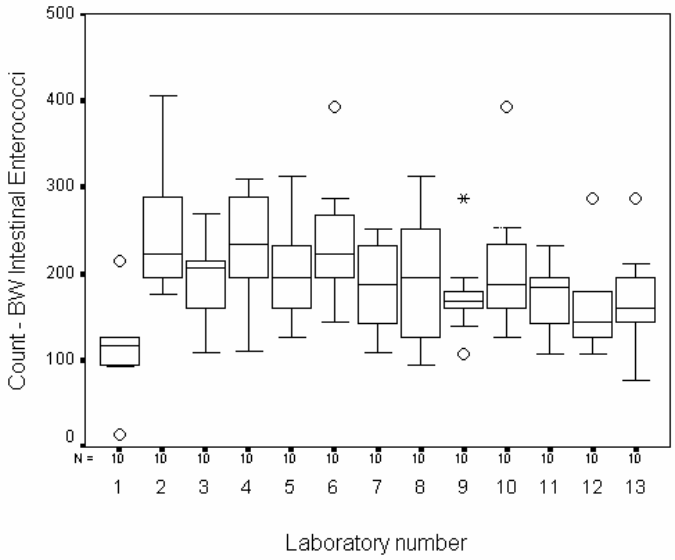


Pastilles

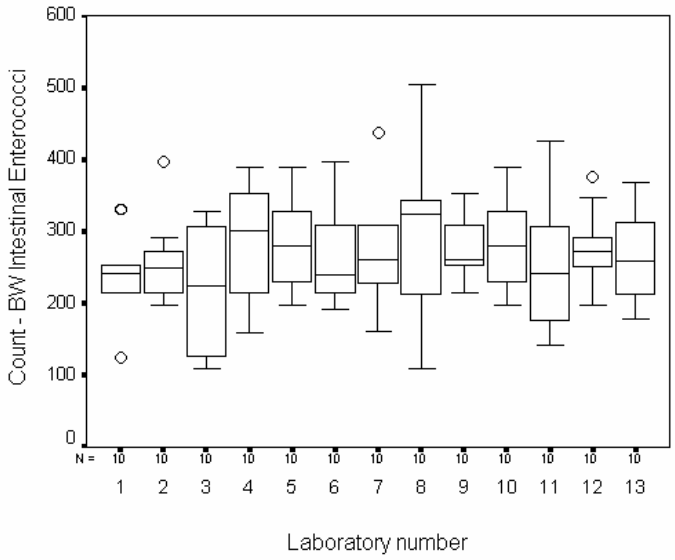


ISO 7899-1 Intestinal *Enterococci,* miniaturised MPN

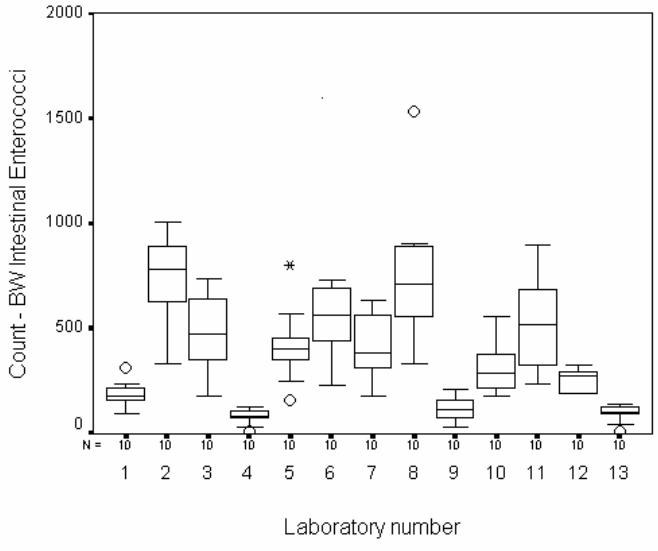
Capsules



Lenticules

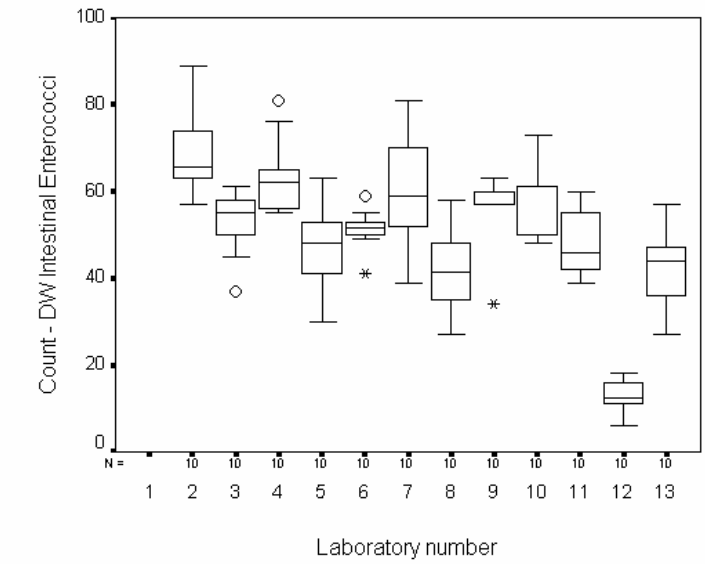


Pastilles



ISO 7899-2 Intestinal *Enterococci*, membrane filtration

Capsules



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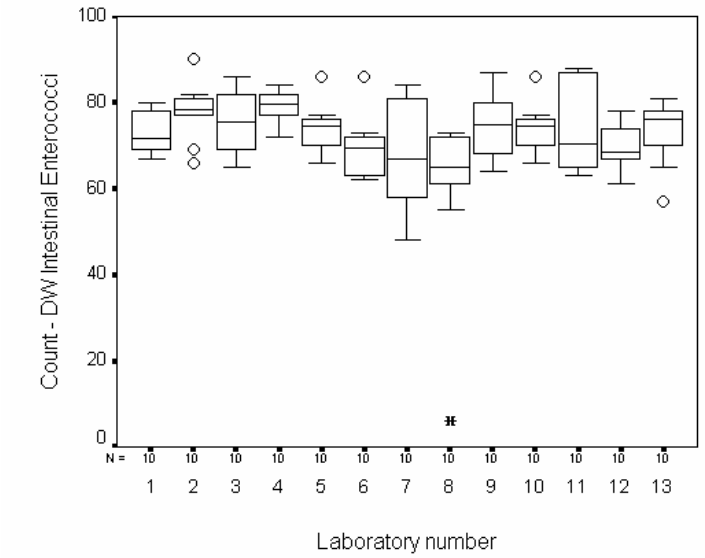

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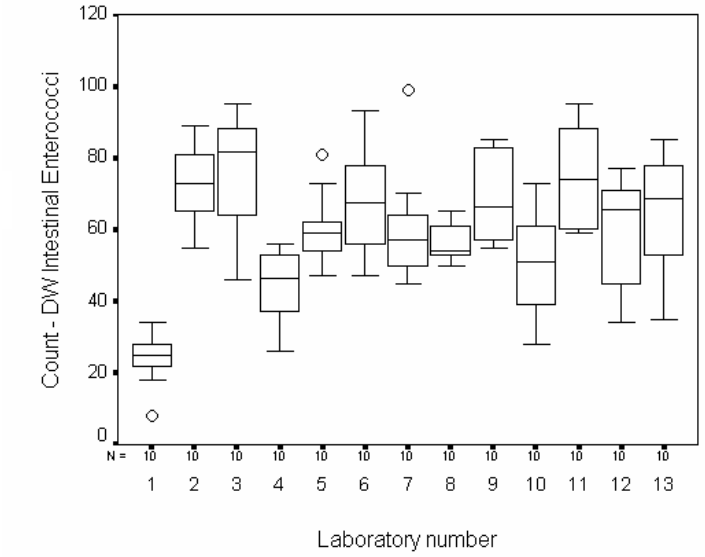
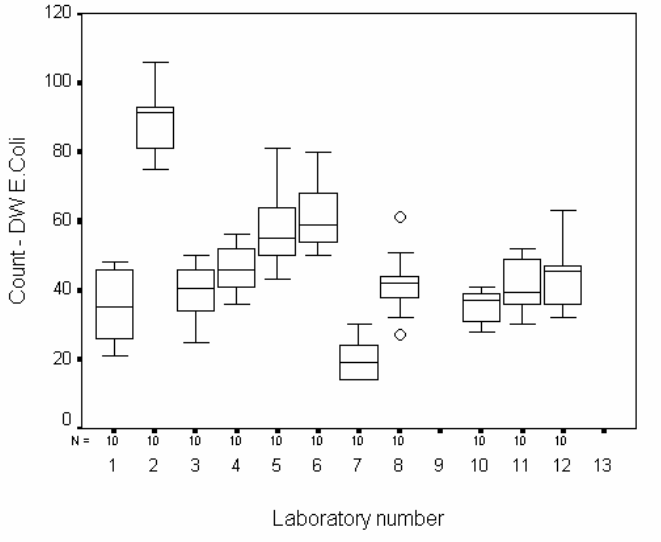


ISO 9308-1 *Escherichia coli* and coliforms, membrane filtration

Capsules



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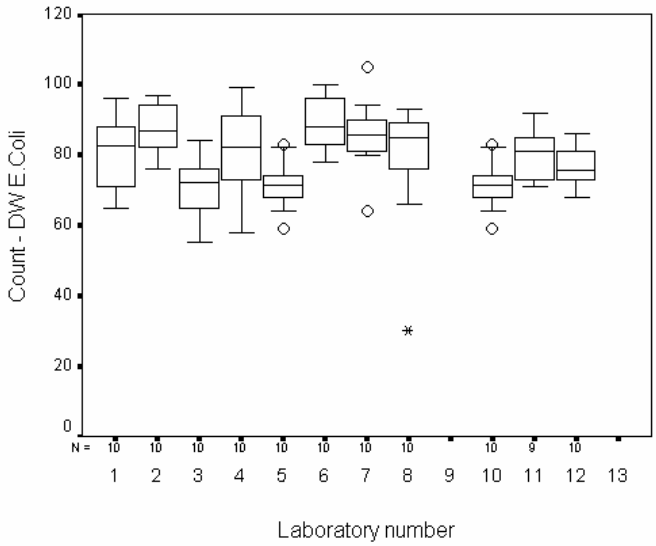

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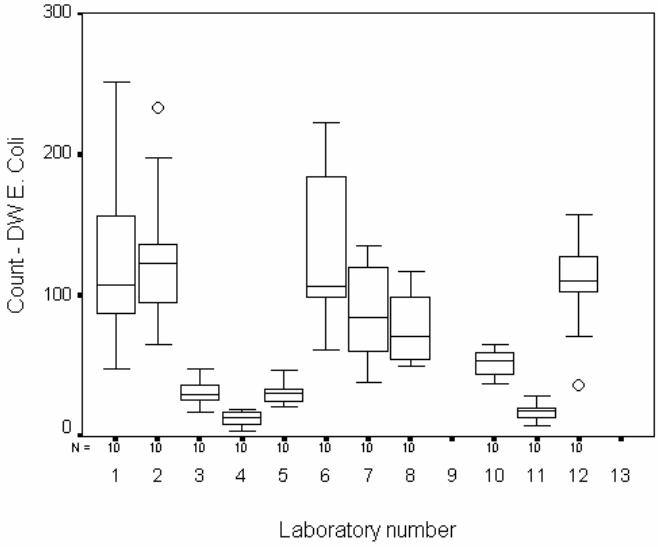
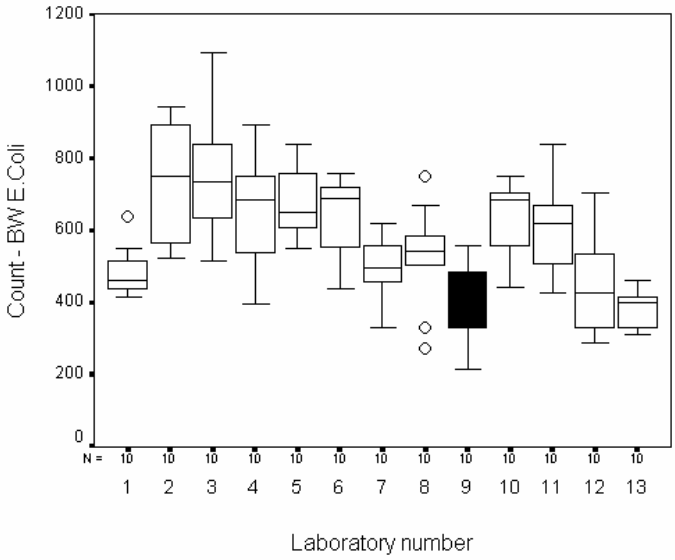
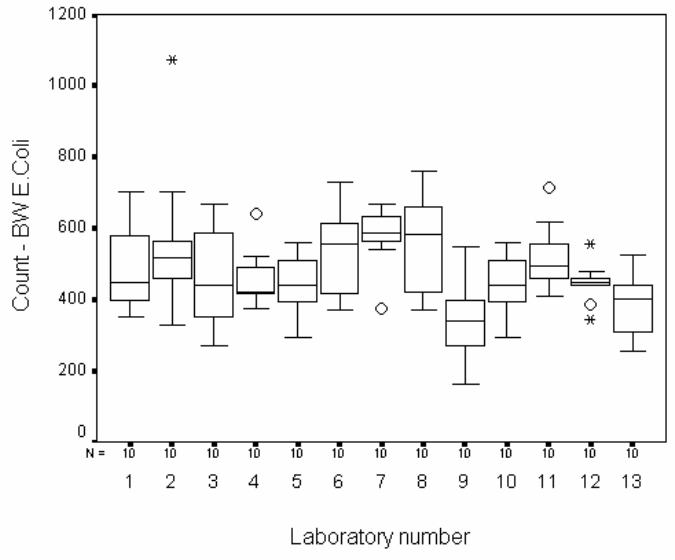


ISO 9308-3 *Escherichia coli*, miniaturised MPN

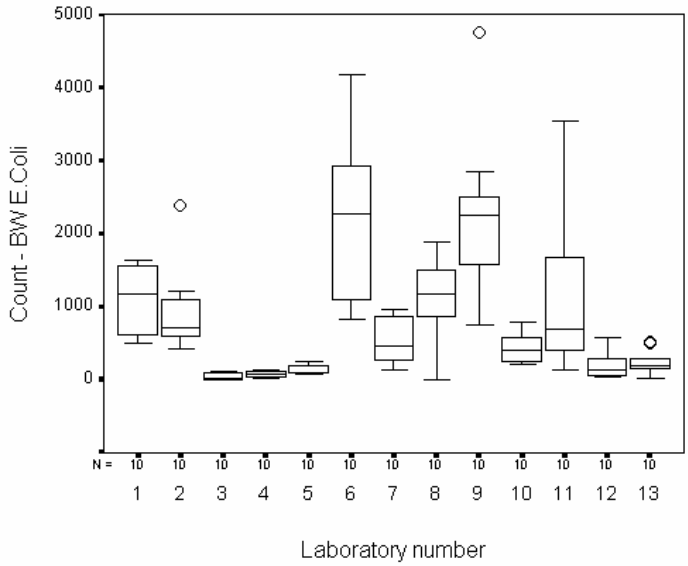
Capsules



Lenticules

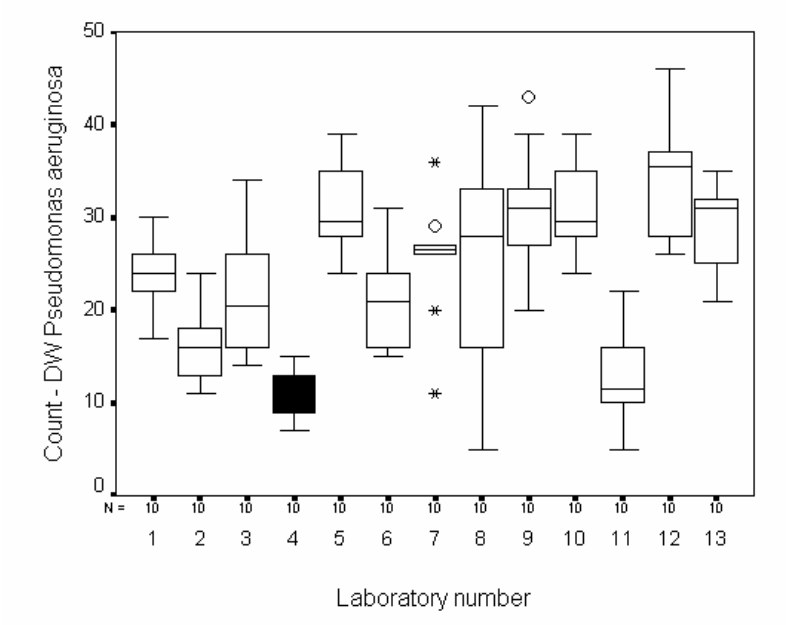


Pastilles

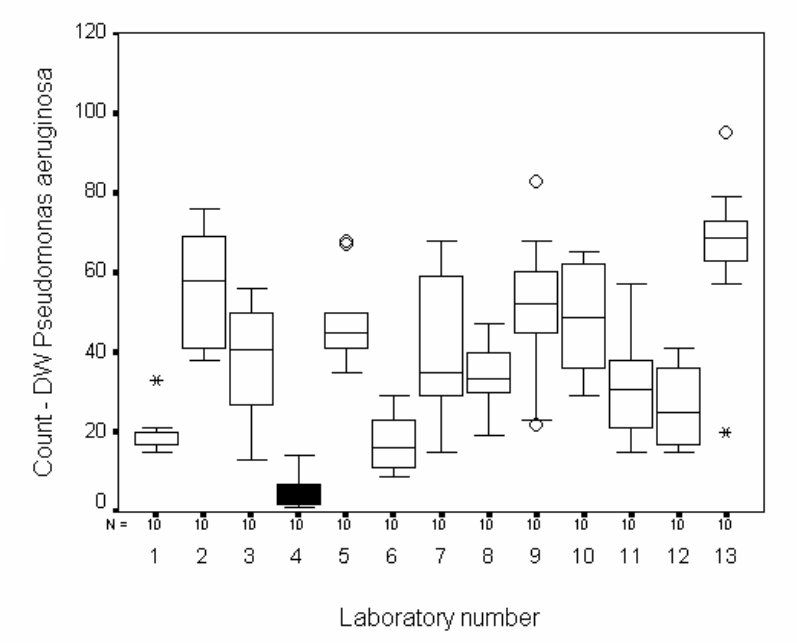


prEN 12780 *Pseudomonas aeruginosa*, membrane filtration

Lenticules



Pastilles



Annex 14 T_1 and T_2 results per laboratory, type of RM and method

*Table A.14.1 T_1 -values of **capsules, lenticules and pastilles** of ISO 6222, Culturable organisms incubated at 22 °C and at 36 °C (accepted data)*

Lab	Capsules				Lenticules				Pastilles			
	22 °C		36 °C		22 °C		36 °C		22 °C		36 °C	
	T_1	T_1/df	T_1	T_1/df	T_1	T_1/df	T_1	T_1/df	T_1	T_1/df	T_1	T_1/df
1	1.81	0.36	4.17	0.83	1.53	0.31	1.37	0.27	0.94	0.19	2.44	0.49
2	6.91	1.38	4.78	0.96	1.74	0.35	15.6	3.12	12.6	2.52	4.06	0.81
3	8.84	1.77	7.13	1.43	1.89	0.38	1.18	0.24	8.87	1.77	0.80	0.16
4	11.2	2.25	2.24	0.45	3.28	0.66	8.04	1.61	17.6	3.53	2.78	0.56
5	2.87	0.57	0.90	0.18	5.77	1.15	5.44	1.09	3.25	0.65	5.13	1.03
6					9.78	1.96	6.86	1.37	19.8	3.96	5.93	1.19
7	5.09	1.02	12.9	2.58								
8	0.85	0.17	5.25	1.05	0.38	0.08	0.50	0.10	0.89	0.18		
9	7.15	1.43	9.05	1.81	2.20	0.44	3.20	0.64	9.52	1.90	1.82	0.36
10	2.01	0.40	2.31	0.46	5.77	1.15	5.44	1.09	7.46	1.49	10.7	2.13
11	2.13	0.43	4.51	0.90	4.46	0.89	2.98	0.60	10.2	2.05	5.65	1.13
12	9.63	1.93	3.42	0.68	2.40	0.48	2.79	0.56	0.87	0.17	4.97	0.99
13	4.77	0.95	2.30	0.46	4.92	0.98	3.30	0.66	2.43	0.49	4.10	0.82

df: degrees of freedom (here 5)

*Table A.14.2 T_1 -values of **capsules** of the membrane filtration methods (accepted data)*

Lab	ISO/WD 6461-2 *Clostridium perfringens*		ISO 7899-2 *Enterococci*		ISO 9308-1 *E. coli* and coliforms	
	T_1	T_1/df	T_1	T_1/df	T_1	T_1/df
1	3.37	0.67			3.71	0.74
2			2.28	0.46	3.13	0.63
3	1.73	0.35	4.06	0.81	6.44	1.29
4	4.32	0.86	1.76	0.35	4.74	0.95
5	8.18	1.64	5.20	1.04	7.37	1.47
6	4.61	0.92	2.48	0.50	11.11	2.22
7			6.41	0.16	7.48	1.50
8	4.05	0.81	11.95	2.39	6.65	1.33
9			5.93	1.19		
10			1.99	0.40	2.71	0.54
11			5.01	1.00	4.57	0.91
12	5.87	1.17	8.15	1.63	15.31	3.06
13			9.02	1.80		

df: degrees of freedom (here 5)

*Table A.14.3 T_2 -values of **capsules** of the different methods, except the miniaturised MPN methods (accepted data)*

Lab	ISO 6222 Culturable org. 22 °C		ISO 6222 Culturable org. 36 °C		ISO/WD 6461-2 *Cl. perfringens*		ISO 7899-1 *Enterococci*		ISO 9308-1 *E. coli* & coliforms	
	T_2	T_2/df	T_2	T_2/df	T_2	T_2/df	T_2	T_2/df	T_2	T_2/df
1	3.03	0.76	2.97	0.74	14.2	3.54			23.8	5.96
2	3.09	0.77	1.74	0.44			8.20	2.05	5.59	1.40
3	5.63	1.41	15.3	3.82	0.62	0.15	5.70	1.42	8.13	2.03
4	13.7	3.42	1.11	0.28	0.86	0.22	8.91	2.23	4.56	1.14
5	0.44	0.11	1.04	0.26	4.75	1.19	12.0	2.99	10.9	2.72
6					8.16	2.04	1.35	0.34	3.25	0.81
7	21.3	5.33	3.24	0.81			19.1	4.77	5.35	1.34
8	27.5	6.87	0.94	0.23	7.34	1.83	13.6	3.41	12.7	3.18
9	5.15	1.29	5.52	1.38			6.12	1.53		
10	2.16	0.54	8.40	2.10			8.91	2.23	3.69	0.92
11	4.17	1.04	5.46	1.36			4.23	1.06	8.31	2.08
12	10.5	2.63	8.01	2.00	8.46	2.12	3.13	0.78	6.42	1.61
13	9.23	2.31	9.44	2.36			9.02	2.25		

df: degrees of freedom (here 4)

*Table A.14.4 T_2 -values of **lenticule**s of the different methods, except the miniaturised MPN methods (accepted data)*

Lab	ISO 6222 Culturable org. 22 °C		ISO 6222 Culturable org. 36 °C		ISO 7899-1 *Enterococci*		ISO 9308-1 *E. coli* & coliforms		prEN 12780 *Ps. aeruginosa*	
	T_2	T_2/df	T_2	T_2/df	T_2	T_2/df	T_2	T_2/df	T_2	T_2/df
1	0.88	0.22	15.5	3.88	2.55	0.28	13.8	1.54	5.95	0.66
2	4.89	1.22	4.19	1.05	5.11	0.57	5.02	0.56	9.90	1.10
3	8.12	2.03	5.80	1.45	5.64	0.63	9.91	1.10	15.4	1.72
4	4.37	1.09	7.57	1.89	1.84	0.20	19.4	2.15		
5	2.42	0.60	5.74	1.44	4.14	0.46	6.94	0.77	6.57	0.73
6	10.1	2.52	5.83	1.46	6.69	0.74	4.77	0.53	12.4	1.38
7					19.0	2.11	11.6	1.28	14.5	1.61
8	9.15	2.29	2.45	0.61	12.0	1.50	40.9	4.54	43.7	4.85
9	18.9	4.72	3.97	0.99	6.82	0.76			14.3	1.59
10	2.42	0.60	5.74	1.44	4.11	0.46	6.94	0.77	6.59	0.73
11	8.14	2.03	0.84	0.21	13.4	1.49	14.6	1.82	19.4	2.15
12	10.3	2.59	9.76	2.44	4.18	0.46	4.39	0.49	12.6	1.40
13	6.20	1.55	6.20	1.55	6.80	0.76			6.62	0.74

df: degrees of freedom (df=4 for culturable organisms, df=9 for the other methods)

*Table A.14.5 T_2 -values of **pastilles** of the different methods, except the miniaturised MPN methods (accepted data)*

Lab	ISO 6222 Culturable org., 22 °C		ISO 6222 Culturable org., 36 °C		ISO/WD 6461-2 *Cl. perfringens*		ISO 7899-1 *Enterococci*		ISO 9308-1 *E. coli* & coliforms		prEN 12780 *Ps. aeruginosa*	
	T_2	T_2/df	T_2	T_2/df	T_2	T_2/df	T_2	T_2/df	T_2	T_2/df	T_2	T_2/df
1	52.3	13.1	10.2	2.56	20.7	2.30	19.2	2.14	290	32.2	10.9	1.21
2	19.5	4.88	18.5	4.64			15.2	1.68	174	19.3	36.3	4.04
3	19.5	488	4.41	1.10	6.71	0.75	32.1	3.56	22.5	2.50	42.4	4.71
4	48.9	12.3	1.92	0.48	13.5	1.50	20.6	2.28	15.0	1.66		
5	1.75	0.44	6.55	1.64	24.8	2.75	14.7	1.63	17.4	1.93	23.0	2.55
6	50.6	12.6	7.90	1.97	30.3	3.37	29.7	3.30	216	24.0	23.8	2.64
7							35.7	3.97	118	13.1	77.6	8.62
8	18.6	4.64			7.40	0.82	4.05	0.45	65.8	7.31	19.0	2.11
9	31.8	7.94	19.8	4.95			20.3	2.25			63.4	6.93
10	20.6	5.14	3.35	0.84	10.6	1.18	36.2	4.02	15.7	1.74	32.8	3.64
11	24.0	5.99	3.55	0.89	23.8	2.64	19.5	2.17	20.0	2.22	50.2	5.58
12	68.4	17.1	9.50	2.38	4.98	0.55	35.2	3.92	91.8	10.2	33.5	3.72
13	145	36.5	39.8	9.95			48.1	5.35			50.1	5.56

df: degrees of freedom (df=4 for culturable organisms, df=9 for the other methods)

Annex 15 Accepted (raw) data of feasibility certification studies

Explanation of the abbreviations in the tables

cult22 (-1,-2): ISO 6222, Culturable organisms, cultured at 22 °C (Anonymous, 1999a), (first replicate, second replicate);
cult36 (-1,-2): ISO 6222, Culturable organisms, cultured at 22 °C (Anonymous, 1999a), (first replicate, second replicate);
clostri (-1, -2): ISO/WD 6461-2, *Clostridium perfringens* (Anonymous, 2001), (first replicate, second replicate);
entmpn (1, 2): ISO 7899-1, Intestinal *Enterococci* miniaturised MPN (Anonymous, 1998a), (first replicate, second replicate);
entmf (-1, -2): ISO 7899-2, Intestinal *Enterococci* membrane filtration (Anonymous, 2000a), (first replicate, second replicate);
colmf (-1, -2): ISO 9308-1, Escherichia coli and coliforms, membrane filtration (Anonymous, 2000b), (first replicate, second replicate);
colmpn (1, 2): ISO 9308-3, Escherichia coli, miniaturised MPN (Anonymous, 1998b), (first replicate, second replicate);
pseudo: prEN 12780, Pseudomonas aeruginosa, membrane filtration (Anonymous, 1999b)
dil 1/2: dilution 1/2 of miniaturised MPN methods (64 wells inoculated)
dil 1/20: dilution 1/2 of miniaturised MPN methods (32 wells inoculated)
mpn: most probable number

*Table A.15.1 Accepted data feasibility certification studies of **capsules**, all methods*

lab	cult22-1	cult22-2	cult36-1	cult36-2	clostri-1	clostri-2	entmpn1	entmpn2	entmf-1	entmf-2	colmf-1	colmf-2	colmpn1	colmpn2
1	49	62	67	67	61	80	110	215			46	48	434	514
1	59	65	75	75	74	65	127	125			21	26	461	442
1	53	53	64	81	87	91	126	93			41	47	549	415
1	63	63	60	76	65	61	124	15			34	36	472	438
1	54	54	59	65	60	61	110	94			21	33	640	461
2	51	62	58	50			270	177	68	65	91	93	565	896
2	52	76	63	64			213	232	63	57	100	90	943	893
2	61	62	59	62			405	179	74	63	80	81	824	824
2	73	64	72	50			215	195	65	66	93	106	534	676
2	73	63	60	56			292	289	76	89	75	92	524	647

lab	cult22-1	cult22-2	cult36-1	cult36-2	clostri-1	clostri-2	entmpn1	entmpn2	entmf-1	entmf-2	colmf-1	colmf-2	colmpn1	colmpn2
3	46	58	33	37	64	68	127	161	58	52	25	38	824	1092
3	61	68	61	55	64	66	270	268	54	37	50	43	514	668
3	57	49	41	63	56	67	109	215	50	45	34	37	633	851
3	53	43	52	67	65	57	215	215	61	56	43	47	633	805
3	41	65	49	47	63	66	197	160	57	60	31	46	661	838
4	74	67	64	62	78	88	292	195	56	62	41	36	397	509
4	56	55	66	54	83	83	177	110	64	59	51	52	683	539
4	101	62	68	68	84	79	234	234	62	55	56	45	690	885
4	51	64	62	69	91	68	251	289	56	65	55	38	750	554
4	65	62	68	59	73	79	309	197	81	76	44	47	736	893
5	54	50	52	50	70	69	215	144	49	63	43	53	800	728
5	50	54	50	56	67	49	127	161	53	54	67	50	619	759
5	44	58	48	50	85	59	194	312	48	36	50	50	565	606
5	48	55	58	54	75	69	161	232	48	48	59	81	838	661
5	50	46	56	50	78	70	292	197	30	41	57	64	640	549
6					75	75	144	232	55	51	54	80	720	697
6					88	73	195	287	52	53	63	50	559	750
6					76	96	215	177	53	41	68	51	438	690
6					70	64	268	393	49	51	59	59	554	759
6					61	70	195	268	59	50	59	76	485	690
7	64	64	72	74			249	251	39	55	23	19	585	465
7	61	73	81	65			143	109	53	67	30	14	353	332
7	59	40	56	66			126	177	63	76	18	14	621	549
7	60	62	49	84			232	197	50	52	14	19	504	559
7	40	35	71	59			215	144	81	70	24	25	457	489
8	84	90	64	43	56	44	94	195	45	28	43	40	559	668
8	85	79	59	58	52	48	144	123	27	35	51	61	504	750
8	69	65	64	54	45	42	270	251	48	38	38	41	585	332
8	60	54	53	55	39	50	312	212	56	35	27	43	554	272
8	52	51	55	60	66	55	126	195	48	58	44	32	529	504
9	69	82	58	70			140	161	61	63				
9	82	70	84	65			195	108	60	59				
9	61	63	73	50			179	177	57	60				
9	66	60	74	63			177	160	60	60				
9	84	58	54	60			161	287	34	57				

lab	cult22-1	cult22-2	cult36-1	cult36-2	clostri-1	clostri-2	entmpn1	entmpn2	entmf-1	entmf-2	colmf-1	colmf-2	colmpn1	colmpn2
10	57	56	56	65			161	177	61	73	34	39	559	676
10	58	69	73	80			127	195	50	49	41	37	750	728
10	58	69	72	70			234	161	48	50	28	39	504	442
10	65	62	71	79			253	393	63	53	28	31	690	704
10	67	69	63	53			234	179	50	50	41	37	705	668
11	48	57	53	68			177	195	55	55	37	30	669	426
11	65	58	58	58			195	161	40	46	47	36	838	529
11	68	61	70	72			108	110	44	42	40	34	609	627
11	54	50	61	46			192	143	49	46	49	51	509	627
11	61	54	58	66			212	232	60	39	39	52	449	764
12	52	72	52	53	51	43	143	127	11	8	36	45	534	705
12	53	66	67	59	55	41	179	108	13	11	46	47	504	332
12	46	37	37	49	64	46	144	144	6	18	63	32	312	442
12	42	61	44	55	64	61	143	179	16	13	63	46	621	412
12	52	45	52	53	65	61	127	287	12	18	45	34	289	353
13	50	57	66	57			179	161	27	45			312	442
13	62	53	53	48			77	77	36	46			332	415
13	62	45	57	46			160	144	43	56			461	415
13	62	73	43	40			161	195	35	42			327	412
13	46	45	61	57			287	212	47	57			386	353

*Table A.15.2 Accepted data feasibility certification studies of **capsules**, miniaturised MPNs (number of positive wells and calculated MPN)*

	entmpn-1			entmpn-2			colmpn-1			colmpn-2		
	no of positive wells			no of positive wells			no. of positive wells			no. of positive wells		
lab	dil 1/2	dil 1/20	MPN-1	dil 1/2	dil 1/20	MPN-2	dil 1/2	dil 1/20	MPN-1	dil 1/2	dil 1/20	MPN-2
1	7	0	110	13	0	215	22	2	434	27	0	514
1	8	0	127	6	2	125	24	1	461	24	0	442
1	7	1	126	5	1	93	26	3	549	22	1	415
1	5	3	124	1	0	15	22	4	472	23	1	438
1	7	0	110	6	0	94	31	1	640	24	1	461
2	15	1	270	10	1	177	29	0	565	37	4	896
2	12	1	213	13	1	232	39	3	943	39	1	893
2	19	4	405	11	0	179	37	1	824	37	1	824
2	13	0	215	11	1	195	27	1	534	30	4	676
2	17	0	292	16	1	289	25	3	524	32	0	647
3	8	0	127	10	0	161	37	1	824	44	1	1092
3	15	1	270	14	2	268	27	0	514	32	1	668
3	6	1	109	13	0	215	30	2	633	35	5	851
3	13	0	215	13	0	215	30	2	633	35	3	805
3	12	0	197	9	1	160	31	2	661	36	3	838
4	17	0	292	11	1	195	22	0	397	26	1	509
4	10	1	177	7	0	110	31	3	683	28	0	539
4	14	0	234	14	0	234	32	2	690	36	5	885
4	14	1	251	16	1	289	34	2	750	27	2	554
4	17	1	309	12	0	197	35	0	736	39	1	893
5	13	0	215	9	0	144	37	0	800	34	1	728
5	8	0	127	10	0	161	31	0	619	35	1	759
5	10	2	194	18	0	312	29	0	565	29	2	606
5	10	0	161	13	1	232	36	3	838	31	2	661
5	17	0	292	12	0	197	31	1	640	26	3	549
6	9	0	144	13	1	232	33	2	720	33	1	697
6	11	1	195	15	2	287	28	1	559	34	2	750
6	13	0	215	10	1	177	23	1	438	32	2	690
6	14	2	268	21	1	393	27	2	554	35	1	759
6	11	1	195	14	2	268	25	1	485	32	2	690

	entmpn-1			entmpn-2			colmpn-1			colmpn-2		
	no of positive wells			no of positive wells			no. of positive wells			no. of positive wells		
lab	dil 1/2	dil 1/20	MPN-1	dil 1/2	dil 1/20	MPN-2	dil 1/2	dil 1/20	MPN-1	dil 1/2	dil 1/20	MPN-2
7	13	2	249	14	1	251	29	1	585	25	0	465
7	8	1	143	6	1	109	20	0	353	19	0	332
7	7	1	126	10	1	177	28	4	621	26	3	549
7	13	1	232	12	0	197	25	2	504	28	1	559
7	13	0	215	9	0	144	23	2	457	26	0	489
8	6	0	94	11	1	195	28	1	559	32	1	668
8	9	0	144	4	4	123	25	1	504	34	2	750
8	15	1	270	14	1	251	29	1	585	19	0	332
8	18	0	312	11	2	212	27	2	554	16	0	272
8	7	1	126	11	1	195	26	2	529	25	2	504
9	5	4	140	10	0	161						
9	11	1	195	5	2	108						
9	11	0	179	10	1	177						
9	10	1	177	9	1	160						
9	10	0	161	15	2	287						
10	10	0	161	10	1	177	28	1	559	30	4	676
10	8	0	127	11	1	195	34	2	750	34	1	728
10	14	0	234	10	0	161	25	2	504	24	0	442
10	15	0	253	21	1	393	32	2	690	31	4	704
10	14	0	234	11	0	179	34	0	705	32	1	668
11	10	1	177	11	1	195	29	5	669	20	4	426
11	11	1	195	10	0	161	36	3	838	26	2	529
11	5	2	108	7	0	110	26	6	609	29	3	627
11	9	3	192	8	1	143	26	1	509	29	3	627
11	11	2	212	13	1	232	21	4	449	33	4	764
12	8	1	143	8	0	127	27	1	534	34	0	705
12	11	0	179	5	2	108	25	2	504	19	0	332
12	9	0	144	9	0	144	18	0	312	24	0	442
12	8	1	143	11	0	179	28	4	621	21	2	412
12	8	0	127	15	2	287	16	1	289	20	0	353
13	11	0	179	10	0	161	18	0	312	24	0	442
13	5	5	77	5	5	77	19	0	332	22	1	415
13	9	1	160	9	0	144	24	1	461	22	1	415
13	10	0	161	11	1	195	17	2	327	21	2	412
13	15	2	287	11	2	212	19	3	386	20	0	353

*Table A.15.3 Accepted data feasibility certification studies of **lenticules**, all methods*

lab	cult22-1	cult22-2	cult36-1	cult36-2	clostri	entmpn	entmf	colmf	colmpn	pseudo
1	57	59	48	49		332	78	66	434	19
1	60	60	60	55		253	74	82	580	24
1	59	69	54	58		251	67	77	704	24
1	53	62	46	47		234	69	71	627	30
1	61	60	79	67		215	78	95	397	26
1						330	71	88	534	26
1						215	68	83	393	28
1						234	72	65	434	22
1						125	80	96	350	17
1						249	71	88	465	22
2	68	76	67	66		253	77	94	1074	18
2	90	83	60	94		251	66	93	397	24
2	67	74	84	76		215	78	87	327	22
2	73	83	69	69		397	79	87	554	17
2	72	70	62	97		197	90	97	529	15
2						272	81	76	565	11
2						197	69	95	461	13
2						292	77	82	504	18
2						249	82	86	480	12
2						230	79	80	704	14
3	56	55	67	72		109	69	78	442	14
3	62	59	53	55		213	76	60	353	16
3	51	39	54	64		234	84	65	438	15
3	64	67	61	63		270	78	76	615	22
3	55	59	52	55		327	86	74	272	26
3						309	69	65	585	21
3						110	74	72	350	26
3						307	82	55	668	20
3						127	75	84	485	20
3						144	65	72	393	34

lab	cult22-1	cult22-2	cult36-1	cult36-2	clostri	entmpn	entmf	colmf	colmpn	pseudo
4	55	59	41	64		253	72	90	480	
4	54	47	61	65		390	73	58	640	
4	62	58	64	68		159	83	73	489	
4	71	55	83	64		270	84	94	419	
4	61	69	63	57		353	78	77	375	
4						215	80	99	415	
4						175	82	91	419	
4						347	81	87	397	
4						353	77	76	519	
4						332	79	66	419	
5	61	53	76	76		289	66	64	509	35
5	56	74	72	73		350	86	70	434	31
5	67	53	54	73		390	73	59	559	35
5	56	51	67	50		215	67	74	438	39
5	54	64	70	66		230	71	68	509	25
5						327	70	83	393	24
5						197	76	74	292	30
5						270	76	68	465	29
5						272	76	82	442	28
5						292	77	73	350	28
6	64	71	48	56		215	72	88	728	16
6	57	38	56	65		312	66	83	539	31
6	69	59	45	64		397	62	100	371	25
6	66	69	48	50		253	86	78	415	20
6	44	67	37	51		192	63	83	554	15
6						230	68	86	612	24
6						251	71	88	712	24
6						215	62	88	415	16
6						309	73	96	559	15
6						195	71	96	585	22

lab	cult22-1	cult22-2	cult36-1	cult36-2	clostri	entmpn	entmf	colmf	colmpn	pseudo
7						309	81	105	640	29
7						309	69	90	565	11
7						228	48	85	591	20
7						213	68	81	539	27
7						270	65	80	612	26
7						438	66	84	633	26
7						253	84	86	585	36
7						287	58	94	375	27
7						161	84	86	565	27
7						251	58	64	668	26
8	49	47	54	59		212		88	633	16
8	51	51	48	49		312	62	89	661	25
8	62	66	52	55		375	62	89	606	25
8	57	53	45	48		327	61	93	419	31
8	65	68	50	53		109	72	82	393	35
8						212	55	76	371	31
8						504	73	66	661	42
8						344	72	91	559	33
8						320	68	30	759	5
8						332	70	80	480	14
9	69	62	63	69		215	72		438	43
9	72	61	55	68		312	68		350	27
9	50	53	64	65		353	64		549	33
9	43	37	75	62		253	78		330	20
9	52	46	51	57		234	68		272	32
9						270	80		397	29
9						253	87		161	30
9						309	79		197	22
9						253	83		390	33
9						287	70		324	39

lab	cult22-1	cult22-2	cult36-1	cult36-2	clostri	entmpn	entmf	colmf	colmpn	pseudo
10	61	53	76	76		289	66	64	509	35
10	56	74	72	73		350	86	70	434	31
10	67	53	54	73		390	73	59	559	35
10	56	51	67	50		215	67	74	438	39
10	54	64	70	66		230	71	68	509	25
10						327	70	83	393	24
10						197	76	74	292	30
10						270	76	68	465	29
10						272	76	82	442	28
10						292	77	73	350	28
11	62	54	63	77		177	73	71	712	10
11	55	75	67	70		212	65	85	415	15
11	62	67	75	64		307	80	81	408	6
11	64	63	69	70		179	68	91	519	22
11	75	85	59	68		304	88	73	619	16
11						141	87		554	16
11						272	63	82	504	5
11						426	64	92	485	13
11						405	65	73	461	10
11						142	87	78	476	10
12	53	65	56	48		270	61	73	554	26
12	42	42	50	49		292	69	68	480	31
12	41	51	37	32		272	62	86	453	28
12	43	41	45	37		252	68	74	461	27
12	39	41	44	35		347	67	74	386	35
12						249	78	71	457	36
12						375	68	80	442	36
12						289	71	81	342	45
12						272	77	86	438	37
12						197	74	77	438	46
13	64	67	65	51		195	65		332	32
13	68	59	68	66		251	78		438	25
13	72	62	63	62		179	70		524	31
13	77	57	56	68		312	57		438	35
13	47	54	70	78		272	81		434	31
13						268	73		307	30
13						213	75		412	23
13						215	79		287	33
13						350	77		393	32
13						368	78		253	21

*Table A.15.4 Accepted data feasibility certification studies of **lenticules**, miniaturised MPNs (number of positive wells and calculated MPN)*

	entmpn			colmpn		
	no. of positive wells			no. of positive wells		
lab	dil 1/2	dil 1/20	MPN	dil 1/2	dil 1/20	MPN
1	19	0	332	22	2	434
1	15	0	253	28	2	580
1	14	1	251	31	4	704
1	14	0	234	29	3	627
1	13	0	215	22	0	397
1	18	1	330	27	1	534
1	13	0	215	21	1	393
1	14	0	234	22	2	434
1	6	2	125	19	1	350
1	13	2	249	25	0	465
2	15	0	253	41	5	1074
2	14	1	251	22	0	397
2	13	0	215	17	2	327
2	22	0	397	27	2	554
2	12	0	197	26	2	529
2	16	0	272	29	0	565
2	12	0	197	24	1	461
2	17	0	292	25	2	504
2	13	2	249	24	2	480
2	12	2	230	31	4	704
3	6	1	109	24	0	442
3	12	1	213	20	0	353
3	14	0	234	23	1	438
3	15	1	270	27	5	615
3	17	2	327	16	0	272
3	17	1	309	29	1	585
3	7	0	110	19	1	350
3	16	2	307	32	1	668
3	8	0	127	25	1	485
3	9	0	144	21	1	393

	entmpn			colmpn		
	no. of positive wells			no. of positive wells		
lab	dil 1/2	dil 1/20	MPN	dil 1/2	dil 1/20	MPN
4	15	0	253	24	2	480
4	20	2	390	31	1	640
4	8	2	159	26	0	489
4	15	1	270	23	0	419
4	20	0	353	21	0	375
4	13	0	215	22	1	415
4	8	3	175	23	0	419
4	18	2	347	22	0	397
4	20	0	353	24	4	519
4	19	0	332	23	0	419
5	16	1	289	26	1	509
5	19	1	350	22	2	434
5	20	2	390	28	1	559
5	13	0	215	23	1	438
5	12.	2	230	26	1	509
5	17	2	327	21	1	393
5	12.	0	197	17	0	292
5	15	1	270	25	0	465
5	16	0	272	24	0	442
5	17	0	292	19	1	350
6	13	0	215	34	1	728
6	18	0	312	28	0	539
6	22	0	397	20	1	371
6	15	0	253	22	1	415
6	9	3	192	27	3	554
6	12	2	230	30	1	612
6	14	1	251	32	3	712
6	13	0	215	22	1	415
6	17	1	309	28	1	559
6	11	1	195	29	1	585
7	17	1	309	31	1	640
7	17	1	309	29	0	565
7	11	3	228	30	0	591
7	12	1	213	28	0	539
7	15	1	270	30	1	612
7	23	1	438	30	2	633
7	15	0	253	29	1	585
7	15	2	287	21	0	375
7	10	0	161	29	0	565
7	14	1	251	32	1	668

lab	entmpn no. of positive wells dil 1/2	dil 1/20	MPN	colmpn no. of positive wells dil 1/2	dil 1/20	MPN
8	11	2	212	30	2	633
8	18	0	312	31	2	661
8	21	0	375	29	2	606
8	17	2	327	23	0	419
8	6	1	109	21	1	393
8	11	2	212	20	1	371
8	25	2	504	31	2	661
8	11	1	344	28	1	559
8	18	1	320	35	1	759
8	19	1	332	24	2	480
9	13	0	215	23	1	438
9	18	0	312	19	1	350
9	20	0	353	26	3	549
9	15	0	253	18	1	330
9	14	0	234	16	0	272
9	15	1	270	22	0	397
9	15	0	253	10	0	161
9	17	1	309	12	0	197
9	15	0	253	20	2	390
9	15	2	287	16	3	324
10	16	1	289	26	1	509
10	19	1	350	22	2	434
10	20	2	390	28	1	559
10	13	0	215	23	1	438
10	12	2	230	26	1	509
10	17	2	327	21	1	393
10	12	0	197	17	0	292
10	15	1	270	25	0	465
10	16	0	272	24	0	442
10	17	0	292	19	1	350

	entmpn			colmpn		
	no. of positive wells			no. of positive wells		
lab	dil 1/2	dil 1/20	MPN	dil 1/2	dil 1/20	MPN
11	10	1	177	32	3	712
11	11	2	212	22	1	415
11	16	2	307	20	3	408
11	11	0	179	24	4	519
11	15	3	304	31	0	619
11	6	3	141	27	2	554
11	16	0	272	25	2	504
11	20	4	426	25	1	485
11	19	4	405	24	1	461
11	7	2	142	23	3	476
12	15	1	270	27	2	554
12	17	0	292	24	2	480
12	16	0	272	22	3	453
12	14	1	252	24	1	461
12	18	2	347	29	3	386
12	13	2	249	23	2	457
12	21	0	375	24	0	442
12	16	1	289	16	4	342
12	16	0	272	23	1	438
12	12	0	197	23	1	438
13	11	1	195	19	0	332
13	14	1	251	23	1	438
13	11	0	179	21	8	524
13	18	0	312	23	1	438
13	16	0	272	22	2	434
13	14	2	268	16	2	307
13	12	1	213	21	2	412
13	13	0	215	15	2	287
13	19	1	350	21	1	393
13	19	2	368	15	0	253

*Table A.15.5 Accepted data feasibility certification studies of **pastilles**, all methods*

lab	cult22-1	cult22-2	cult36-1	cult36-2	clostri	entmpn	entmf	colmf	colmpn	pseudo
1	12	13	14	16	5	160	30	156	1294	21
1	39	45	15	20	6	179	23	115	1599	33
1	38	42	7	7	4	215	28	48	1638	20
1	22	22	12	17	7	94	18	103	1160	20
1	49	44	9	13	1	234	27	184	1007	20
1					3	177	26	49	1183	17
1					6	312	8	112	509	15
1					10	177	22	96	1567	20
1					4	179	34	251	574	20
1					14	110	24	87	612	16
2	74	85	11	12		627	88	233	434	52
2	79	80	18	19		893	70	114	1049	40
2	49	79	11	14		800	71	136	640	38
2	68	56	15	15		332	75	197	591	41
2	40	59	34	20		465	65	108	606	76
2						1007	81	134	415	69
2						767	89	131	791	76
2						981	55	95	1092	46
2						728	61	65	1202	67
2						791	77	93	2383	64
3	34	36	14	12	10	419	80	26	93	27
3	61	60	17	20	6	736	88	30	0	41
3	61	44	16	17	10	353	64	28	0	13
3	40	46	11	12	11	434	92	29	15	50
3	47	73	15	12	10	640	66	17	46	27
3					14	585	95	36	0	56
3					10	332	58	21	15	55
3					10	177	83	30	15	40
3					8	514	88	48	110	45
3					16	736	46	37	94	40

lab	cult22-1	cult22-2	cult36-1	cult36-2	clostri	entmpn	entmf	colmf	colmpn	pseudo
4	51	67	12	11	13	77	48	19	46	
4	89	46	11	13	7	30	49	9	110	
4	25	34	10	10	8	110	56	4	77	
4	35	37	17	11	5	109	26	9	77	
4	38	33	11	17	3	94	53	15	61	
4					3	126	33	11	109	
4					8	127	37	14	144	
4					7	15	45	17	94	
4					3	77	55	12	46	
4					7	77	39	17	15	
5	85	101	9	11	9	375	58	21	76	43
5	84	101	8	9	2	253	52	47	110	50
5	83	84	17	16	9	161	81	34	197	45
5	91	98	7	18	4	390	59	28	253	40
5	90	87	14	14	8	568	47	33	197	68
5					11	438	54	25	197	67
5					6	412	62	25	76	41
5					5	800	59	34	234	45
5					12	457	60	34	94	35
5					19	353	73	23	176	45
6	69	41	21	16	18	647	48	222	1086	14
6	51	32	15	19	26	565	47	103	2341	29
6	95	72	14	19	9	728	62	78	2194	10
6	32	50	26	23	17	690	56	184	1317	17
6	37	47	8	18	6	442	70	109	824	23
6					8	504	73	99	1104	15
6					16	234	83	110	3114	25
6					6	697	78	61	4179	18
6					12	232	93	100	2873	9
6					8	559	65	202	2929	11
7						179	60	60	872	29
7						312	54	68	127	59
7						565	64	84	270	36
7						619	50	38	633	68
7						438	52	135	287	24
7						393	70	128	896	29
7						234	63	84	956	66
7						633	50	52	126	56
7						375	99	111	529	34
7						330	45	120	393	15

lab	cult22-1	cult22-2	cult36-1	cult36-2	clostri	entmpn	entmf	colmf	colmpn	pseudo
8	48	52			6	647	57	117	920	36
8	54	57			9	559	50	84	1076	31
8	60	55			6	791	55	65	30	47
8	79	75			15	904	53	55	1793	30
8	71	78			11	893	53	104	861	41
8					9	539	53	74	1884	24
8					10	1531	61	50	1502	30
8					9	782	63	99	1264	19
8					13	612	65	50	1502	40
8					12	332	53	68		38
9	31	50	17	13		92	85		2496	68
9	49	34	7	8		144	56		4753	45
9	57	61	18	14		77	85		2322	59
9	26	29	5	8		61	59		2444	45
9	24	35	20	19		143	55		2843	55
9						213	62		2182	49
9						94	83		1382	83
9						30	57		1647	22
9						161	74		1579	60
9						160	71		742	23
10	61	59	21	16	7	215	46	59	442	36
10	81	51	13	21	7	195	35	59	253	29
10	84	84	26	18	9	253	67	52	215	64
10	80	88	22	15	11	504	73	55	568	62
10	58	53	32	16	16	330	61	65	253	50
10					12	375	58	44	215	44
10					5	330	51	37	791	47
10					11	559	51	55	353	65
10					11	215	28	37	453	31
10					6	177	39	49	654	52
11	85	70	9	12	11	393	60	19	861	57
11	90	107	21	12	13	434	73	14	412	15
11	53	72	17	15	4	234	88	13	126	29
11	83	101	11	14	11	633	88	26	430	30
11	58	77	18	10	7	896	75	18	661	31
11					16	270	95	29	734	48
11					11	893	73	13	1677	16
11					24	324	59	18	272	21
11					7	606	78	8	1838	35
11					13	683	60	20	3543	38

lab	cult22-1	cult22-2	cult36-1	cult36-2	clostri	entmpn	entmf	colmf	colmpn	pseudo
12	46	50	15	9	18	195	70	109	292	17
12	79	86	25	20	13	195	69	157	197	40
12	76	80	15	23	15	312	71	103	46	19
12	44	39	20	24	16	330	56	36	109	25
12	34	34	12	17	22	266	40	128	141	15
12					20	292	77	112	61	16
12					18	292	62	108	574	41
12					12	287	45	115	353	28
12					18	195	74	71	61	25
12					16	270	34	127	77	36
13	95	86	9	11		15	76		161	67
13	37	39	31	22		46	35		94	63
13	25	34	7	13		110	53		213	20
13	104	97	12	16		143	78		158	73
13	38	43	30	30		94	74		292	79
13						110	36		15	70
13						144	55		253	70
13						93	83		514	57
13						94	85		509	65
13						126	63		160	95

*Table A.15.6 Accepted data feasibility certification studies of **pastilles**, miniaturised MPNs (number of positive wells and calculated MPN)*

	entmpn			colmpn		
	no. of positive wells			no. of positive wells		
lab	dil 1/2	dil 1/20	MPN	pos2	pos20	MPN3
1	9	1	160	44	8	1294
1	11	0	179	52	3	1599
1	13	0	215	52	4	1638
1	6	0	94	43	5	1160
1	14	0	234	42	1	1007
1	10	1	177	46	1	1183
1	18	0	312	26	1	509
1	10	1	177	51	4	1567
1	11	0	179	27	3	574
1	7	0	110	30	1	612
2	29	3	627	22	2	434
2	39	1	893	43	1	1049
2	37	0	800	31	1	640
2	19	0	332	30	0	591
2	25	0	465	29	2	606
2	42	1	1007	22	1	415
2	36	0	767	36	1	791
2	42	0	981	44	1	1092
2	34	1	728	47	0	1202
2	36	1	791	58	7	2383
3	23	0	419	5	1	93
3	35	0	736	0	0	0
3	20	0	353	0	0	0
3	22	2	434	1	0	15
3	31	1	640	3	0	46
3	29	1	585	0	0	0
3	19	0	332	1	0	15
3	10	1	177	1	0	15
3	27	0	514	7	0	110
3	35	0	736	6	0	94

	entmpn			colmpn		
	no. of positive wells			no. of positive wells		
lab	dil 1/2	dil 1/20	MPN	pos2	pos20	MPN3
4	5	0	77	3	0	46
4	2	0	30	7	0	110
4	7	0	110	5	0	77
4	6	1	109	5	0	77
4	6	0	94	4	0	61
4	7	1	126	6	1	109
4	8	0	127	9	0	144
4	1	0	15	6	0	94
4	4	1	77	3	0	46
4	5	0	77	1	0	15
5	21	0	375	3	2	76
5	15	0	253	7	0	110
5	10	0	161	12	0	197
5	20	2	390	15	0	253
5	26	4	568	12	0	197
5	23	1	438	12	0	197
5	21	2	412	3	2	76
5	37	0	800	14	0	234
5	23	2	457	6	0	94
5	20	0	353	9	2	176
6	32	0	647	40	7	1086
6	29	0	565	59	4	2341
6	34	1	728	57	6	2194
6	32	2	690	48	2	1317
6	24	0	442	37	1	824
6	25	2	504	43	3	1104
6	14	0	234	61	9	3114
6	33	1	697	63	11	4179
6	13	1	232	60	9	2873
6	28	1	559	61	7	2929

	entmpn			colmpn		
	no. of positive wells			no. of positive wells		
lab	dil 1/2	dil 1/20	MPN	pos2	pos20	MPN3
7	11	0	179	37	3	872
7	18	0	312	8	0	127
7	29	0	565	15	1	270
7	31	0	619	30	2	633
7	23	1	438	15	2	287
7	21	1	393	37	4	896
7	14	0	234	38	5	956
7	30	2	633	7	1	126
7	21	0	375	26	2	529
7	18	1	330	21	1	393
8	32	0	647	37	5	920
8	28	1	559	43	2	1076
8	36	1	791	2	0	30
8	40	0	904	55	2	1793
8	39	1	893	36	4	861
8	28	0	539	56	2	1884
8	51	3	1531	50	4	1502
8	35	2	782	47	2	1264
8	30	1	612	50	4	1502
8	19	0	332	0	0	
9	3	3	92	60	4	2496
9	9	0	144	64	10	4753
9	5	0	77	58	6	2322
9	3	1	61	58	8	2444
9	8	1	143	61	6	2843
9	12	1	213	56	8	2182
9	6	0	94	48	4	1382
9	2	0	30	50	8	1647
9	10	0	161	49	8	1579
9	9	1	160	33	3	742

	entmpn			colmpn		
	no. of positive wells			no. of positive wells		
lab	dil 1/2	dil 1/20	MPN	pos2	pos20	MPN3
10	13	0	215	24	0	442
10	11	1	195	15	0	253
10	15	0	253	13	0	215
10	25	2	504	26	4	568
10	18	1	330	15	0	253
10	21	0	375	13	0	215
10	18	1	330	36	1	791
10	28	1	559	20	0	353
10	13	0	215	22	3	453
10	10	1	177	30	3	654
11	21	1	393	36	4	861
11	22	2	434	21	2	412
11	14	0	234	7	1	126
11	30	2	633	21	3	430
11	37	4	896	31	2	661
11	15	1	270	32	4	734
11	39	1	893	52	5	1677
11	16	3	324	16	0	272
11	29	2	606	55	3	1838
11	31	3	683	62	10	3543
12	11	1	195	17	0	292
12	11	1	195	12	0	197
12	18	0	312	3	0	46
12	18	1	330	6	1	109
12	13	3	266	6	3	141
12	17	0	292	4	0	61
12	17	0	292	27	3	574
12	15	2	287	20	0	353
12	12	1	195	4	0	61
12	15	1	270	5	0	77

	entmpn			colmpn		
	no. of positive wells			no. of positive wells		
lab	dil 1/2	dil 1/20	MPN	pos2	pos20	MPN3
13	1	0	15	10	0	161
13	3	0	46	6	0	94
13	7	0	110	12	1	213
13	8	1	143	7	3	158
13	6	0	94	17	0	292
13	7	0	110	1	0	15
13	9	0	144	15	0	253
13	5	1	93	23	5	514
13	6	0	94	22	6	509
13	7	1	126	9	1	160