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MICROCRM: Preparation and control of batches of microbiological reference materials consisting of capsules

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Abstract

Batches of microbiological reference materials (RMs) were prepared for performing feasibility certification studies in the European project 'MICROCRM'. Each of the three partners in the project, Institute Pasteur (Lille, Fr), the Public Health Laboratory Services, (Newcastle, UK) and the National Institute for Public Health and the Environment, (Bilthoven, NL) produced batches of one of three different types of microbiological RMs (pastilles, lenticules or capsules). Four batches of capsule RMs, each containing a different strain, were prepared at the RIVM. Each batch was tested for its homogeneity, long-term stability at storage temperature (-20 °C) and short-term stability at elevated temperatures (5 °C, 22 °C, 30 °C, and 36 °C). The batch of capsule RMs containing *Escherichia coli* was analysed using 2 different culture methods (ISO 9308-1 and ISO 9308-3). The batch of RMs containing *Enterococcus faecium* was analysed using 4 different culture methods (ISO 6222, 22 °C and 36 °C, ISO 7899-1 and ISO 7899-2). Batches of RMs containing *Clostridium perfringens* and *Pseudomonas aeruginosa* were each analysed using one culture method (ISO/WD 6461-2 and prEN 12780, respectively). The batch of RMs containing *P. aeruginosa* showed poor stability at storage temperature (-20 °C), therefore it was decided not to use this batch for the feasibility certification studies. The quality of the three other batches of capsule RMs was sufficient to warrant use in further studies.

Contents

Abbreviations and symbols

BPW	Buffered peptone water
cfp	colony forming particles
CIP	Collection de l'Institute Pasteur
hcmp	highly contaminated milk powder
IPL	Institute Pasteur in Lille
KFA	Kenner faecal agar
LSA	Laurylsulphate agar
LTTC	Lactose TTC agar with Tergitol 7
MGB	Microbiological Laboratory for Health Protection
MPN	Most Probable Number
NCTC	National Collection of Type Cultures
PHLS	Public Health Laboratory Service
RIVM	National Institute for Public Health and the Environment
RM	Reference material
rpm	rotations per minute
S&B	Slanetz and Bartley medium
TSA	Tryptone Soya Agar
TSC	Tryptose Cycloserine agar
YA	Yeast Extract agar

Samenvatting

Op 1 februari 2001 ging het Europese project ‘MICROCRM’ van start. De afkorting MICROCRM staat voor (vertaald): ‘Microbiologische gecertificeerde referentiematerialen ter ondersteuning van EU waterwetgeving, testen van laboratorium performance en kwaliteitscontrole’. Het doel van het project is om de condities vast te stellen welke nodig zijn om belangrijke microbiologische referentiematerialen (RMs) te produceren en te certificeren ter ondersteuning van EU waterwetgeving (Drinkwaterrichtlijn en Zwemwaterrichtilijn).
De drie partners in het project (Instituut Pasteur in Lille, Fr; Public Health Laboratory Service Newcastle, UK; Rijksinstituut voor Volksgezondheid en Milieu Bilthoven, NL) kwamen overeen om elk partijen van één van drie verschillende typen microbiologische RMs te produceren (pastilles, lenticules of capsules). Aan het begin van het project zijn tussen de drie partners afspraken gemaakt over de criteria waaraan iedere partij RMs moet voldoen (selectie van micro-organismen, besmettingsniveaus, analysemethoden, homogeniteit en stabiliteit). Bij het RIVM werden vier partijen van capsule RMs bereid, elk met een verschillende stam. Iedere partij werd gecontroleerd op homogeniteit, lange-duur stabiliteit bij opslagtemperatuur (-20 °C) en korte-duur stabiliteit bij verhoogde temperaturen (5 °C, 22 °C, 30 °C en 36 °C). De partij capsule RMs met *Escherichia coli* werd geanalyseerd met twee verschillende kweekmethoden (ISO 9308-1 en ISO 9308-3). De partij RMs met *Enterococcus faecium* werd geanalyseerd met vier verschillende kweekmethoden (ISO 6222, 22 °C en 36 °C, ISO 7899-1 and ISO 7899-2). Partijen RMs met *Clostridium perfringens* en *Pseudomonas aeruginosa* werden ieder geanalyseerd met één kweekmethode (ISO/WD 6461-2 en prEN 12780 respectievelijk). De partij RMs met *Pseudomonas aeruginosa* was niet stabiel bij opslagtemperatuur (-20 °C) en besloten werd om deze partij niet te gebruiken voor de haalbaarheid certificeringsstudies. De kwaliteit van de andere partijen capsule RMs was voldoende om gebruikt te worden in verdere studies.

Summary

On 1 February 2001 the European project 'MICROCRM' started. The acronym MICROCRM stands for: 'Microbiological Certified Reference Materials in support of EU water legislation, performance testing and laboratory control'. The aim of the project is to determine the conditions that are necessary to produce and certify key water microbiological reference materials (RMs) that will support EU water legislations (Drinking Water and Bathing Water Directives).
The three partners in the project, Institute Pasteur (Lille, Fr), the Public Health Laboratory Services (Newcastle, UK) and the National Institute for Public Health and the Environment (Bilthoven, NL) agreed to produce each batches of one of three different types of microbiological RMs (pastilles, lenticules or capsules). At the start of the project agreements were made with the three partners about the criteria each batch of RMs should meet (selection of micro-organisms, concentration levels, methods for analyses, homogeneity and stability).
Four batches of capsule RMs, each containing a different strain, were prepared at the RIVM. Each batch was tested for its homogeneity, long-term stability at storage temperature (-20 °C) and short-term stability at elevated temperatures (5 °C, 22 °C, 30 °C, and 36 °C). The batch of capsule RMs containing *Escherichia coli* was analysed using two different culture methods (ISO 9308-1 and ISO 9308-3). The batch of RMs containing *Enterococcus faecium* was analysed using four different culture methods (ISO 6222, 22 °C and 36 °C, ISO 7899-1 and ISO 7899-2). Batches of RMs containing *Clostridium perfringens* and *Pseudomonas aeruginosa* were each analysed using one culture method (ISO/WD 6461-2 and prEN 12780, respectively). The batch of RMs containing *P. aeruginosa* showed poor stability at storage temperature (-20 °C), therefore it was decided not to use this batch for the feasibility certification studies. The quality of the other batches of capsule RMs was sufficient to warrant use in further studies.

1. Introduction

The European project 'MICROCRM' started on 1 February 2001. 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 is to determine the conditions that are necessary to produce and certify key water microbiological reference materials (RMs) that will support EU Water legislations (Drinking Water and Bathing Water Directives).
It was agreed that the three partners in the project would each produce 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), would produce capsules;
- Public Health Laboratory Service (PHLS) Board, Newcastle, United Kingdom, would produce lenticules;
- Institute Pasteur of Lille (IPL), France; Water & Environment Department, would produce pastilles.

During a meeting organised on 4-6 March 2001, held in Lille (France), agreements were made on:
- micro-organisms;
- concentration levels;
- methods for analyses;
- homogeneity test;
- stability studies.

All information was summarised in the report on WP1 (Mooijman *et al*., 2001) and was used for preparing and controlling the different batches of RMs. The relevant part of the report on WP1 is given in Annex 2.

In the period June – December 2001 all batches of RMs were prepared by the 3 partners. The homogeneity of all batches was controlled and stability studies (short-term and long-term) were started immediately after preparation of each batch. The long-term stability studies lasted until December 2002.

This report describes the preparation and results of the batches of RMs prepared by RIVM-MGB, consisting of capsules.

2. Materials and Methods

2.1 Materials

Test strains
The following test strains were used for the preparation of the batches of capsules:
- *Escherichia coli* (WR1; NCTC 13167);
- *Enterococcus faecium* (WR63; NCTC 13169);
- *Clostridium perfringens* (D10; NCTC 13170);
- *Pseudomonas aeruginosa* (CIP 82118).

The results of the biochemical determination of the test strains are given in Annex 1.

Sterile milk powder
Spray-dried skim milk powder of Friesche Vlag (Leeuwarden, the Netherlands) was used, γ-irradiated with a dose of 10 kGy. Batch no. 8716200028455 (production year 1996).

Capsules
Gelatin capsules no. 1 of Eli Lilly (Indianapolis, USA) were used, γ-irradiated with a dose of 10 kGy. Different colour combinations (upper side/lower side capsule) were used for the different strains.
- blue/blue: *Escherichia coli*
- pink/pink: *Enterococcus faecium*
- green/white: *Clostridium perfringens*
- white/white: *Pseudomonas aeruginosa*

Evaporated milk
Sterile evaporated half-fat milk (‘Halvamel’) was used of Friesche Vlag (Leeuwarden, the Netherlands). Milk fat: 4%; dry mass (fat free): 20%; water: 76%.

Apparatus
- Spray-drier: Niro Mobil Minor Atomizer;
- Melamine mortar, with nominal volume of 3 litres, and pestle, sterilised by autoclaving at 121 °C for 15 min.;
- Mixing apparatus: Willy A. Bachofen Maschinfabriken AG, type Turbula Type 10B (mixing speed used: 22 rpm);
- Aluminium capsule filling apparatus (for 60 capsules). The apparatus was cleaned with hot water and ethanol (80%) and dried for at least 15 h at 80 °C before use;
- Mixing vessel: stainless steel drum with a nominal volume of 17 litres, sterilised for 15 min at 121 °C.

2.2 Production process

The preparation and control of the capsule RMs include the following steps.
A selected test strain is cultured, concentrated and mixed with sterile evaporated milk. The milk suspension is spray-dried to produce the 'highly contaminated milk powder' (hcmp). A small amount of the hcmp is mixed with sterile milk powder in small steps of equal amounts (as much as possible). Up to a total of *ca* 450 g powder is mixed in a pharmaceutical way, using a mortar and pestle. During each mixing step, the powder is mixed with the pestle for 20 sec, followed by remodelling using a paper card. This procedure is repeated 3 times per mixing step. Larger amounts (>450 g) of powders are mixed in a mixing apparatus (1 hour per mixing step). The powders are mixed until the desired concentration level is reached.
Next the mixed powder is filled into gelatin capsules (0.2 – 0.3 g per capsule). Before filling the complete mixture, a test batch of 60 capsules is filled and controlled for concentration level and homogeneity. The concentration level would differ per batch of RMs, depending on the test strain and depending on the intended use. Homogeneity of the test batch is tested as described in paragraph 2.3. If the pre-set criteria for the contamination level and homogeneity are fulfilled more capsules are filled with the mixed powder, to finalise the preparation of the batch.
The homogeneity of the filling of the capsules is checked by weighing approximately one capsule of every filling of 60 capsules. A homogeneous filled batch should fulfil the following criterion: $(s / \bar{x}).100\% < 3\%$. Where $\bar{x}$ is the mean weight and s is its standard deviation.
The final batch is again tested for homogeneity (see 2.3) and for stability (see 2.4).
Hcmp, mixed powders and filled capsules are stored at (-20 ± 5) °C.

2.3 Homogeneity studies

During the project it was decided to test the homogeneity of the RMs in two ways:

1. Test for Poisson distribution by calculating T_1 and T_2 (Annex 2 and 3).
   T_1 is a measure of the variation within capsules and should follow a χ^2-distribution with I (=the number of tested capsules) degrees of freedom. If T_1 follows a Poisson distribution, the value of T_1/I should be *ca* 1. T_2 is a measure of the variation between capsules. Homogeneity of a batch of capsules would be considered acceptable if $T_2/ (I-1) \leq 2$.
2. Measure of reproducibility, T.
   T is defined as 'the value below which the ratio (max/min) of two random counts of capsules is found with 95% probability'. Information on calculation of T is given in Annex 3. Homogeneity of a batch of capsules would be considered acceptable if the maximum ratio $T \leq 3$. However, the aim was $T \leq 2$.

The results of the tests for the Poisson distribution (T_1, T_2) are dependent of the contamination level, whereas the test for reproducibility (T) is not. The test for Poisson distribution is (in case of RMs) the more 'classical' way of testing homogeneity. The

usefulness of both homogeneity studies was evaluated during the project. Details on statistical analyses concerning the homogeneity studies are given in Annex 3.

2.4 Stability studies

The stability of the final batches of RMs was tested in two ways:

1. Long-term stability at storage temperature.
   The storage temperature of the capsule RMs is (-20 ± 5) °C. To check the long-term stability of the batch, every month 5 capsules were enumerated in duplicate with the relevant method for at least 1 year.
2. Short-term stability at elevated temperatures.
   The influence of elevated temperatures on the stability of the batch of RMs was also tested. For this purpose some units (capsules) of 1 batch of RMs were stored at four different temperatures (5 °C, 22 °C, 30 °C and 36 °C). Daily, twice a week, weekly or more (depending on the decay rate) 5 capsules were enumerated in duplicate with the relevant method for at maximum 6 months. More details on the 'plan' of the stability study at elevated temperatures is given in Annex 2.

The main demand concerning stability of a batch of RMs is that it would be stable at –20 °C (± 5 °C) for preferably 1 year.
For the long-term stability studies as well as for the short-term stability studies instability is assumed if the counts fall significantly over time. In case of instable materials, the half-life was calculated. Half-life is defined as the estimate of the time for half of the organisms to die. This value can be estimated from the reliability curve associated with the Weibull distribution (Weibull, 1951). More information on the statistical analyses of the stability studies is given in Annex 4.

2.5 Preparation and control of the capsule RMs

For the preparation of the different batches of capsule RMs, highly contaminated milk-powders (hcmp's) were used which were spray-dried in earlier projects. However, for *Pseudomonas aeruginosa* no hcmp was present, so that this needed to be prepared during the project (see 2.5.4)

2.5.1 *Escherichia coli* (WR1; NCTC 13167)

The RMs containing *Escherichia coli* were analysed using two methods: ISO 9308-1 (Anonymous, 2000b) and ISO 9308-3 (Anonymous, 1998b). For this purpose the mean contamination level in the capsules was aimed to lie between 600 and 1500 cfp/capsule. Three grams of hcmp batch 6-2 (spray-dried in 1993) was mixed in 7 steps of (almost) equal amounts with sterile skim milk powder until a total of 450 g mixed powder. A test batch of

60 capsules was filled on 25 June 2001 and 10 capsules were enumerated in duplicate by means of membrane filtration, following the procedures as described in Annex 5 and 6. As the medium of ISO 9308-1 (Anonymous, 2000), Lactose TTC agar with sodium heptadecylsulphate (Tergitol-7) (LTTC), was not immediately available, the concentration level and homogeneity was checked on Laurylsulphate agar (LSA; Anonymous, 1990). As the test batch fulfilled the pre-set criteria, the final batch of *ca* 2000 capsules was made on 19 and 20 July 2001, obtaining the batch number: 6-2-25/06/01. The homogeneity of the filling of the capsules was checked by weighing in total 33 capsules.
The capsules were packed in urine containers (nominal volume 20 ml), with 5 capsules per container, together with a small bag of desiccant. Capsules were stored at (-20 ± 5) °C. Homogeneity of the final batch was checked on 33 capsules on LSA (Anonymous, 1990) in July 2001, on 30 capsules on LTTC (Anonymous, 2000b) in October 2001 and on 30 capsules on 'microtitre plates' (Anonymous, 1998b) in November 2001. Long-term stability study started soon after finalising the batch. Short-term stability studies at elevated temperatures started in March 2002 on LTTC and on 'microtitre plates'.

2.5.2 *Enterococcus faecium* (WR63; NCTC 13169)

The RMs containing *Enterococcus faecium* were analysed using three methods: ISO 6222 (Anonymous, 1999a), ISO 7899-1 (Anonymous 1998a) and ISO 7899-2 (Anonymous, 2000a). For this purpose the mean contamination level in the capsules was aimed to lie between 250 and 750 cfp/capsule.
One gram of hcmp batch LWL34 (spray-dried in 1991) was mixed in 7 steps of (almost) equal amounts with sterile milk powder until a total of 100 g mixed powder. One gram of this latter mixed powder was further mixed in 10 steps of (almost) equal amounts with sterile milk powder until a total of 1000 g final mixed powder. A test batch of 60 capsules was prepared on 24 July 2001 and 20 capsules were enumerated in duplicate by means of membrane filtration, following the procedures as described in Annex 5 and 6. As the medium of ISO 7899-2 (Anonymous, 2000a), Slanetz and Bartley medium (S&B), was not immediately available, the concentration level and homogeneity was checked on Kenner Faecal agar (KFA, Anonymous, 1984). As the test batch fulfilled the pre-set criteria, the final batch of *ca* 3500 capsules was made on 3, 4 and 5 September 2001, obtaining batch number LWL34-24/07/01. The homogeneity of the filling of the capsules was checked by weighing in total 30 capsules.
The capsules were packed and stored as described for *E. coli*. Homogeneity of the final batch was checked on 30 capsules on S&B (Anonymous, 2000a) in September 2001, on Yeast Extract agar (YA), incubated at 22 °C and at 36 °C (Anonymous, 1999a) in October 2001 and on 'microtitre pates' (Anonymous, 1998a) in November 2001. Long-term stability study started soon after finalising the batch. Short-term stability studies at elevated temperatures started in December 2001 on YA, incubated at 36 °C (Anonymous, 1999a.) and started in June 2002 on S&B, on YA incubated at 22 °C and on 'microtitre plates'.

2.5.3 *Clostridium perfringens* (D10; NCTC 13170)

The RMs containing *Clostridium perfringens* were analysed using one method: ISO(WD) 6461-2 (Anonymous, 2001). The mean contamination level in the capsules was aimed to lie between 250 and 750 cfp/capsule.
Five grams of hcmp batch LWL35 (spray-dried in 1991) was mixed in 6 steps of (almost) equal amounts with sterile skim milk powder until a total of 315 g mixed powder (LWL3501). Three grams of LWL3501 was further mixed in 7 steps of (almost) equal amounts with sterile skim milk powder until a total of 300 g final mixed powder. A test batch of 60 capsules was prepared on 24 October 2001 and 10 capsules were enumerated in duplicate by means of membrane filtration, following the procedures as described in Annex 5 and 6. The concentration level and homogeneity was checked on Tryptose Cycloserine agar (TSC; Anonymous, 2001). As the test batch fulfilled the pre-set criteria, the final batch of *ca* 1200 capsules was made on 1 November 2001, obtaining batch number LWL3501-24/10/01. The homogeneity of the filling of the capsules was checked by weighing in total 20 capsules. The capsules were packed and stored as described for *E. coli*. Homogeneity of the final batch was checked on 30 capsules on TSC (Anonymous, 2001) in November 2001. Long-term stability study started soon after finalising the batch. Short-term stability studies on TSC at elevated temperatures started in January 2002.

2.5.4 *Pseudomonas aeruginosa* (CIP 82118)

The RMs containing *Pseudomonas aeruginosa* analysed using one method: prEN 12780 (Anonymous, 1999b). The mean contamination level in the capsules was aimed to lie between 250 and 750 cfp/capsule.
For *Pseudomonas aeruginosa* no highly contaminated milkpowder was available. Therefore a milk suspension containing the relevant strain was spray-dried in November 2001. For this purpose, the test strain *Pseudomonas aeruginosa* (CIP 82118) was cultured, while shaken at 100 rpm, in 25 flasks each containing 30 ml Buffered Peptone Water (BPW)[1], at (37 ± 1) °C for (48 ± 4) h. Next the culture was centrifuged (4000 g, 20 min), the supernatant removed and the pellet resuspended (with glass beads) in 3 L sterile evaporated milk and mixed carefully. The mean number of colony forming particles (cfp) of *Pseudomonas aeruginosa* in the milk suspension was determined by spreading 0.1 ml of 10-fold dilutions (prepared in peptone saline solution, Annex 5) on Tryptone Soya agar plates (TSA; Anonymous, 2000b), incubated at (37 ± 1) °C for (48 ± 4) h.
The milk suspension was spray-dried at an inlet temperature of 200 °C and an outlet temperature of 70-75 °C. The resulting hcmp obtained batch number LWL36 and was stored in plastic bags at (-20 ± 5) °C. Twenty five grams of hcmp batch LWL36 was mixed in three steps of (almost) equal amounts with sterile skim milkpowder until a total of 250 g mixed powder. A test batch of 60 capsules was filled on 15 November 2001 and 10 capsules were

enumerated in duplicate by means of membrane filtration (on two different dates), following the procedures as described in Annex 5 and 6. The concentration level and homogeneity was checked on CN-agar (Anonymous, 1999b). Although it seemed that the mean level was not fully stable, still the final batch of *ca* 1000 capsules was made on 11 December 2001, obtaining batch number LWL36-21/11/01. The homogeneity of the filling of the capsules was checked by weighing in total 20 capsules. After finalising the batch the homogeneity was checked on 10 capsules on CN-agar. Furthermore, it was checked whether the batch was sufficiently stable for use by analysing 5-10 capsules in duplicate on five different dates (of which 3 dates in one month period). Different volumes (2, 3 or 4 ml) of the capsule solutions were analysed in accordance with the procedures described in Annex 5 & 6.

[1] BPW contains (g/L): peptone, 10.0; sodium chloride, 5.0; disodium hydrogen phosphate, 4.5 and potassium dihydrogen phosphate 1.5. Sterilised for 15 min at 121 °C.

3. Results

3.1 *Escherichia coli* (WR1; NCTC 13167)

The pre-set criteria for the batch of RMs containing *Escherichia coli* were:
- mean contamination level: between 600 and 1500 cfp/capsule;
- T_1 not significantly from a χ^2-distribution with I (= the number of tested capsules) degrees of freedom;
- $T_2/ (I-1) \leq 2$;
- $T \leq 3$ (preferably ≤ 2).

In Table 1 the results of the test batch are given. As each capsule was reconstituted in 10 ml peptone saline solution (see Annex 5) and of each solution 0.5 ml was enumerated according to Annex 6 on LSA, using the old version of ISO 9308-1 (Anonymous, 1990), the counted mean level was expected to lie between 30 and 75 cfp.
The test batch fulfilled the pre-set criteria so that the final batch was prepared. The capsules were homogeneously filled as s / $\bar{x}$ was 1% ($\bar{x}$ = 0.291 g and s = 0.004 g).
The homogeneity of the final batch was tested on LSA (Anonymous, 1990), as well as on LTTC (Anonymous, 2000) as on the microtitre plates (Anonymous, 1998b). The results are given in Table 1.
Although the test batch had fulfilled the pre-set criteria, the final batch tested on LSA was just at the limits of the criteria (mean concentration as well as variation between capsules when tested with T_2). The final batch tested on LTTC and on the microtitre plates fulfilled well the criterion of the mean contamination level, but showed high variation between capsules when tested for a Poisson distribution (T_2). However, the result of the ratio T fulfilled the (more strict) criterion ($T \leq 2$).
The raw data of all homogeneity test results are given in Annex 7.

The long-term stability studies started in October 2001 on LTTC (ISO 9308-1; Anonymous, 2000) and in November 2001 on the microtitre plates (ISO 9308-3, Anonymous, 1998b). Results are given in Figure 1, respectively Figure 2. In these figures the individual (5) capsule results (duplicate means) are indicated as dots and a line is drawn through the mean result per measurement point. Also the results of the homogeneity test is indicated ('hom', 30 capsules). The results of the long-term stability studies showed stable results for both methods (ISO 9308-1 and ISO 9308-3) for the period measured (13-14 months).
Results of the short-term stability studies at elevated temperatures are given in Figure 3 for the study on LTTC and in Figure 4 for the study on the microtitre plates. For both methods the batch of *Escherichia coli* RMs appeared to be unstable at elevated temperatures. The estimated half-lifes are given in Table 2 (further details are given in Annex 4).

The raw data of the stability studies are given in Annex 8 (long-term) and in Annex 9 (short-term).

Table 1 Results of homogeneity tests of test batch and final batch (6-2-25/06/01) of capsules containing Escherichia coli.

	I	J	Mean cfp[4]	T_1[5]	T_2/ (I-1)	T
Test batch on LSA[1]						
Tested June 2001	10	2	56.8	7.6	1.0	nt
Final batch on LSA[1]						
Tested July 2001	33	2	35.6	43.6	2.4	nt
Final batch on LTTC[2]						
Tested Oct. 2001	30	2	51.1	26.1	4.3	1,8
Final batch on microtitre plates[3] . Tested Nov. 2001	30	2	5.3	29.6	3.8	2.1

[1]: ISO 9308-1: 1990, LSA: Laurylsulphate agar; [2]: ISO 9308-1: 2000, LTTC: Lactose TTC agar with sodium heptadecylsulphate; [3]: ISO 9308-3: 1998.
I: number of tested capsules; J: number of tested replicates.
[4]: On LSA and on LTTC, 0.5 ml of the reconstituted capsule solution (10 ml) was enumerated. The mean number of cfp counted on the filters is given. On the microtitre plates the result is given as mean MPN/ml.
[5]: Upper limits of the χ^2 distribution: 18.3 (at I=10); 47.4 (at I=33) and 43.8 (at I=30).
nt: not tested

Table 2 Estimated half-lifes calculated from the results of the short-term stability study at elevated temperatures for capsules containing Escherichia coli, batch 6-2-25/06/01

Storage	Estimated half-life (days)	
temperature (°C)	ISO 9308-1 (LTTC)	ISO 9308-3 (microtitre)
5	60	41
22	20	39
30	10	8
36	6	8

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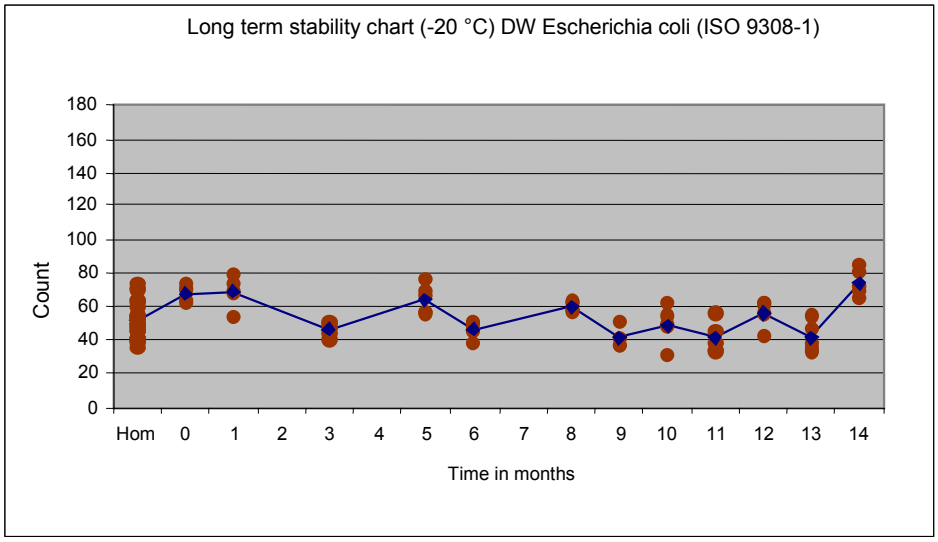

Figure 1 *Long-term stability study of batch of capsules 6-2-25/06/01, containing Escherichia coli, stored at –20 °C. Every dot indicates the duplicate mean of individual capsule results being 30 capsules for the homogeneity study (hom) and 5 capsules for the stability study, enumerated in duplicate on LTTC (ISO 9308-1; Anonymous, 2000). Start study 31 October 2001.*

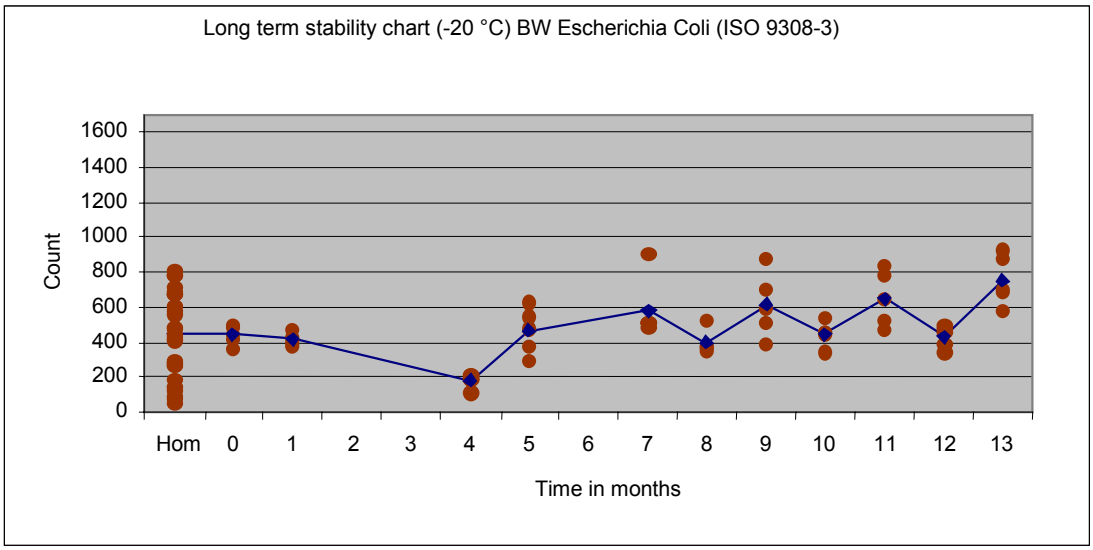

Figure 2 *Long-term stability study of batch of capsules 6-2-25/06/01, containing Escherichia coli, stored at –20 °C. Every dot indicates the duplicate mean of individual capsule results being 30 capsules for the homogeneity study (hom) and 5 capsules for the stability study, enumerated in duplicate on microtitre plates (ISO 9308-3; Anonymous, 1998b). Start study 19 November 2001.*

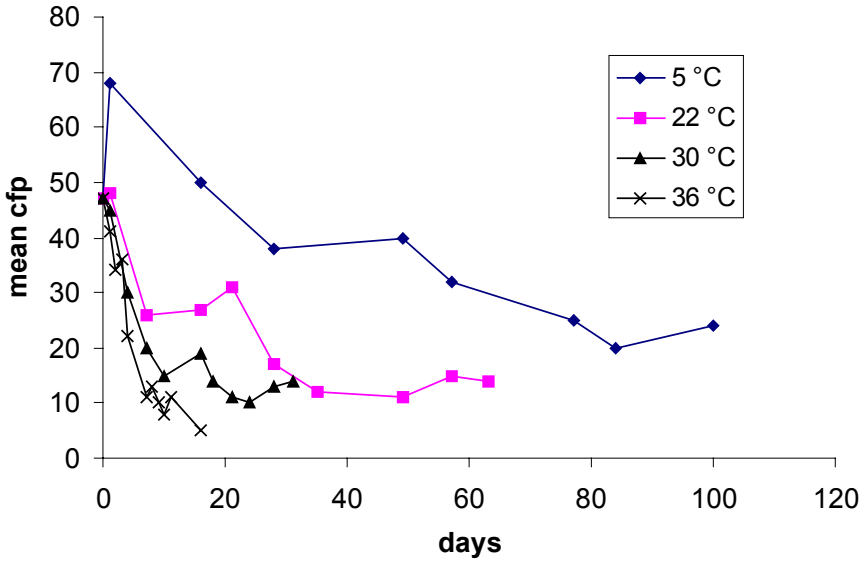

Figure 3 *Short-term stability study at elevated temperatures of batch of capsules 6-2-25/06/01, containing Escherichia coli. Every measurement point gives mean number of 5 capsules enumerated in duplicate on LTTC (ISO 9308-1; Anonymous, 2000). Start study 18 March 2001.*

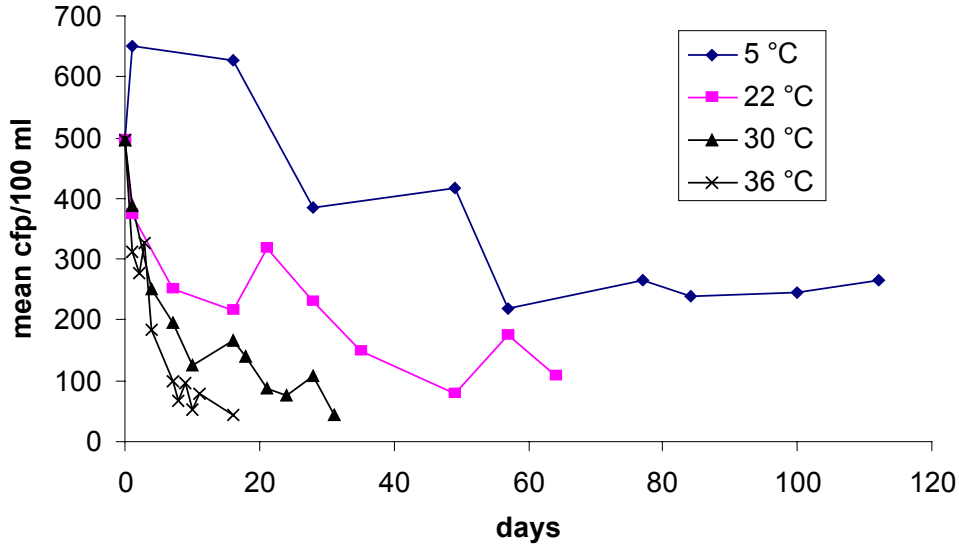

Figure 4 *Short-term stability study at elevated temperatures of batch of capsules 6-2-25/06/01, containing Escherichia coli. Every measurement point gives mean number of 5 capsules enumerated in singular on microtitre plates (ISO 9308-3; Anonymous, 1998b). Start study 18 March 2001.*

3.2 *Enterococcus faecium* (WR63; NCTC 13169)

The pre-set criteria for the batch of RMs containing *Enterococcus faecium* were:
- mean contamination level: between 250 and 750 cfp/capsule;
- T_1 not significantly from a χ^2-distribution with I (= the number of tested capsules) degrees of freedom;
- $T_2/ (I-1) \leq 2$;
- $T \leq 3$ (preferably ≤ 2).

In Table 3 the results of the test batch are given. As each capsule was reconstituted in 10 ml peptone saline solution (see Annex 5) and of each solution 1 ml was enumerated according to Annex 6 on KFA, using the old version of ISO 7899-2 (Anonymous, 1984), the counted mean level was expected to lie between 25 and 75 cfp.
The test batch fulfilled the pre-set criteria so that the final batch was prepared. The capsules were homogeneously filled as s / $\bar{x}$ was 1.7 % ($\bar{x}$ = 0.292 g and s = 0.005 g).
The homogeneity of the final batch was tested on S&B (Anonymous, 2000a), on YA incubated at 22 °C and at 36 °C (Anonymous, 1999a) and on the microtitre plates (Anonymous, 1998a). The results are given in Table 3. The results of all homogeneity studies tested for all methods fulfilled the pre-set criteria.
The raw data of all homogeneity test results are given in Annex 7.

The long-term stability studies started in September 2001 on S&B (ISO 7899-2; Anonymous, 2000a) and in October 2001 on YA, 22 °C and 36 °C (ISO 6222; Anonymous, 1999a) and in November 2001 on the microtitre plates (ISO 7899-1; Anonymous, 1998a). Results are given in respectively Figures 5-8. In these figures the individual (5) capsule results (duplicate means) are indicated as dots and a line is drawn through the mean result per measurement point. Also the results of the homogeneity test is indicated ('hom', 30 capsules). The results of the long-term stability studies showed stable results for all methods for the period measured (13-14 months).

Results of the short-term stability studies at elevated temperatures are given in Figures 9-12. For all methods the batch of capsules containing *Enterococcus faecium* showed stable results at all tested storage temperatures for the period measured. As the materials were stable, no half-lifes were calculated (further details are given in Annex 4).
The raw data of the stability studies are given in Annex 8 (long-term) and Annex 9 (short-term).

Table 3 *Results of homogeneity tests of test batch and final batch (LWL34-24/07/01) of capsules containing Enterococcus faecium.*

	I	J	Mean cfp[5]	T_1[6]	T_2/ (I-1)	T
Test batch on KFA[1]						
Tested July 2001	20	2	54.8	14.3	2.4	nt
Final batch on S&B[2]						
Tested September 2001	30	2	52.0	28.0	0.7	1.3
Final batch on YA, 22 °C[3]						
Tested Oct. 2001	30	2	56.5	24.3	0.7	1.3
Final batch on YA, 36 °C[3]						
Tested Oct. 2001	30	2	55.4	32.1	1.1	1.3
Final batch on microtitre plates[4] . Tested Nov. 2001	30	2	2.0	26.1	1.6	2.1

[1]: ISO 7899-2: 1984, KFA: Kenner Faecal agar; [2]: ISO 7899-2: 2000, S&B: Slanetz and Bartley; [3]: ISO 6222: 1999, YA: Yeast extract agar; [4]: ISO 7899-1: 1998
I: number of tested capsules; J: number of tested replicates; nt: not tested
[5]: On KFA, S&B and on YA, 1 ml of the reconstituted capsule solution (10 ml) was enumerated. The mean number of cfp counted on the filters is given. On the microtitre plates the result is given as mean MPN/ml.
6: Upper limits of the χ^2 distribution: 31.4 (at I=20) and 43.8 (at I=30).

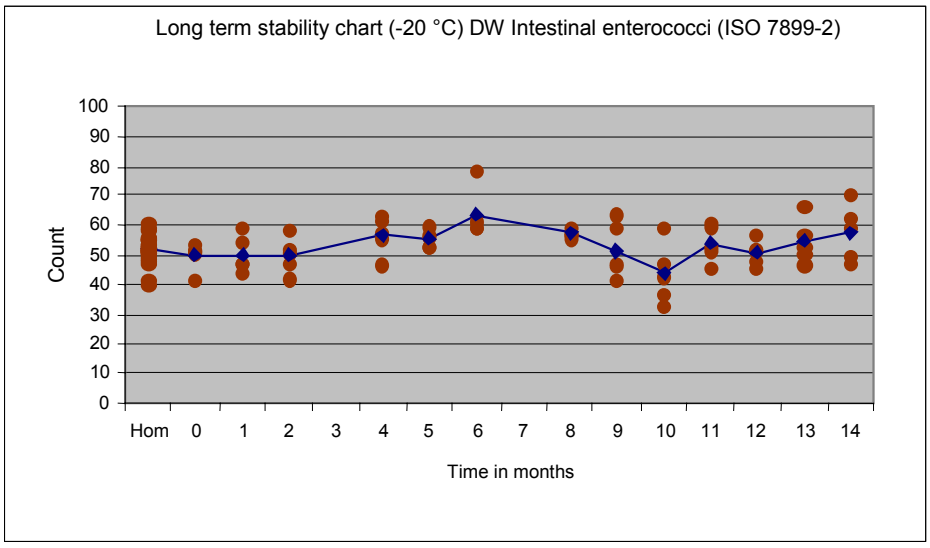

Figure 5 *Long-term stability study of batch of capsules LWL34-24/07/01, containing Enterococcus faecium, stored at –20 °C. Every dot indicates the duplicate mean of individual capsule results being 30 capsules for the homogeneity study (hom) and 5 capsules for the stability study, enumerated in duplicate on S&B (ISO 7899-2; Anonymous, 2000). Start study 24 September 2001.*

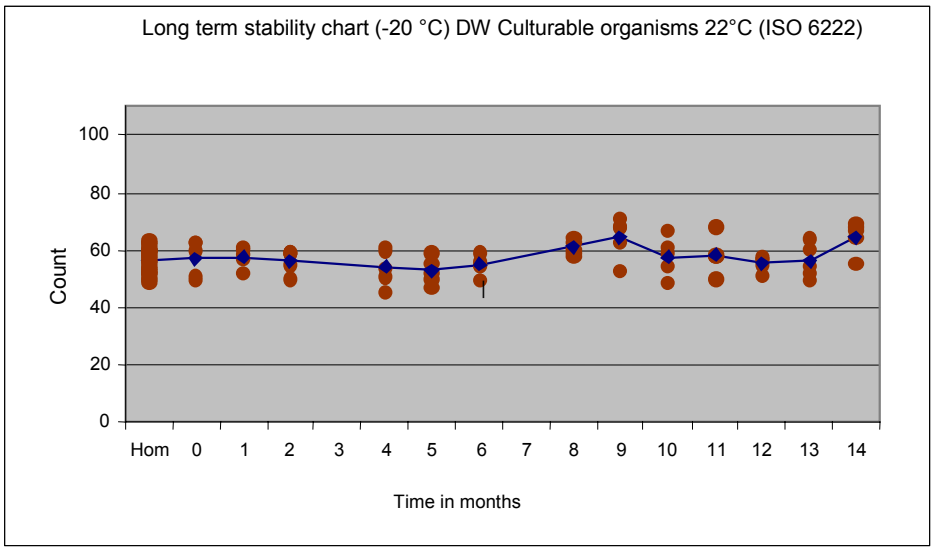

Figure 6 *Long-term stability study of batch of capsules LWL34-24/07/01, containing Enterococcus faecium, stored at –20 °C. Every dot indicates the duplicate mean of individual capsule results being 30 capsules for the homogeneity study (hom) and 5 capsules for the stability study, enumerated in duplicate on YA, 22 °C (ISO 6222; Anonymous, 1999). Start study 15 October 2001.*

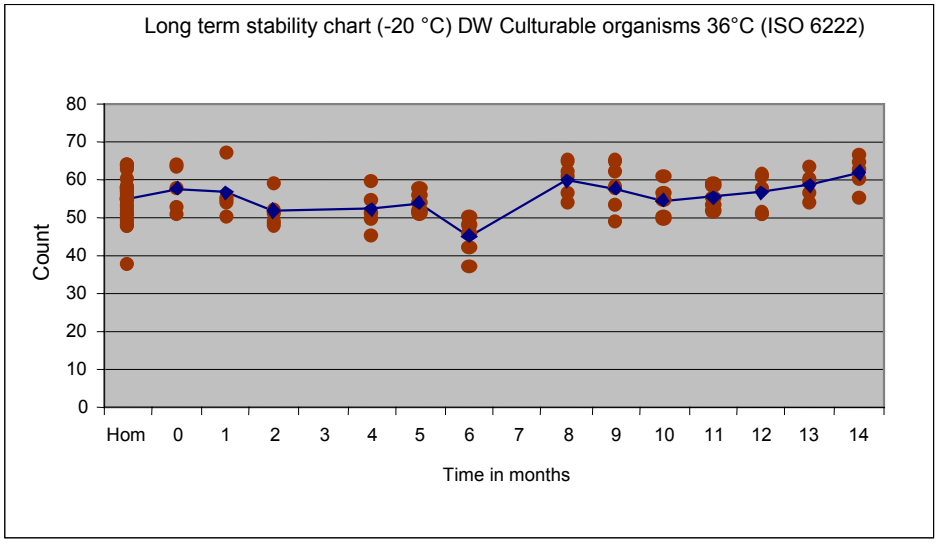

Figure 7 *Long-term stability study of batch of capsules LWL34-24/07/01, containing Enterococcus faecium, stored at –20 °C. Every dot indicates the duplicate mean of individual capsule results being 30 capsules for the homogeneity study (hom) and 5 capsules for the stability study, enumerated in duplicate on YA, 36 °C (ISO 6222; Anonymous, 1999). Start study 15 October 2001.*

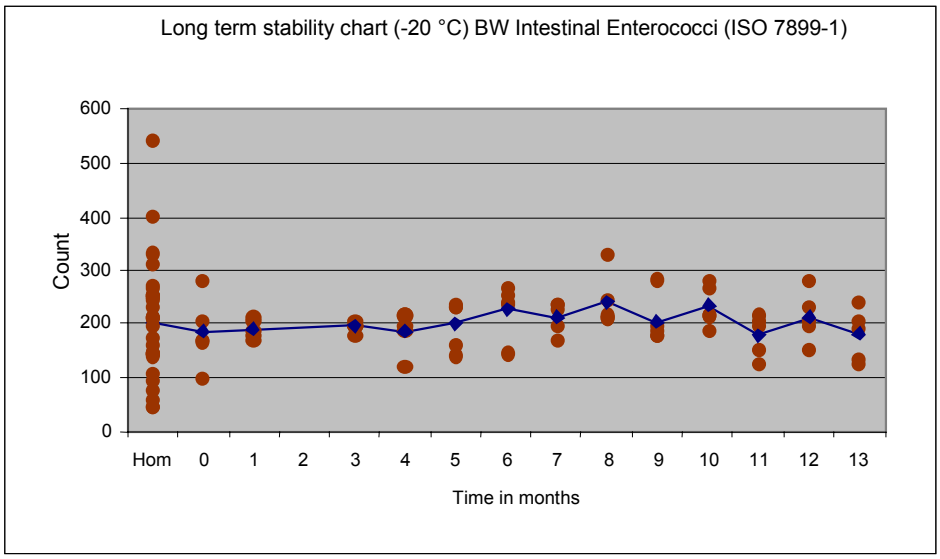

Figure 8 *Long-term stability study of batch of capsules LWL34-24/07/01, containing Enterococcus faecium, stored at –20 °C. Every dot indicates the duplicate mean of individual capsule results being 30 capsules for the homogeneity study (hom) and 5 capsules for the stability study, enumerated in duplicate on microtitre plates (ISO 7899-1; Anonymous, 1998a). Start study 20 November 2001.*

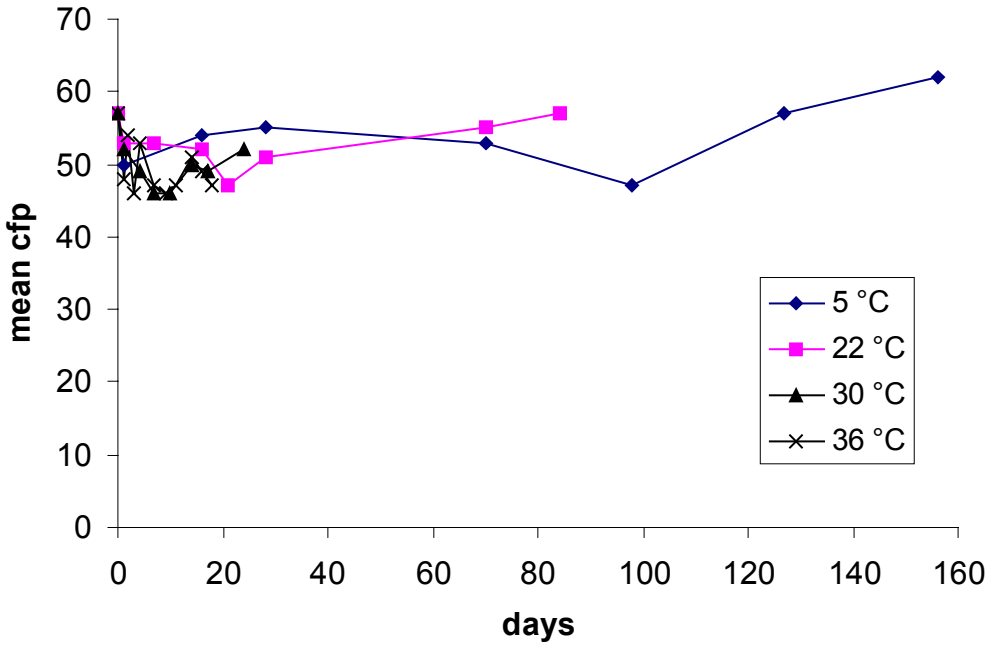

Figure 9 *Short-term stability study at elevated temperatures of batch of capsules LW34-24/07/01, containing Enterococcus faecium. Every measurement point gives mean number of 5 capsules enumerated in duplicate on S&B (ISO 7899-2; Anonymous, 2000). Start study 10 June 2002.*

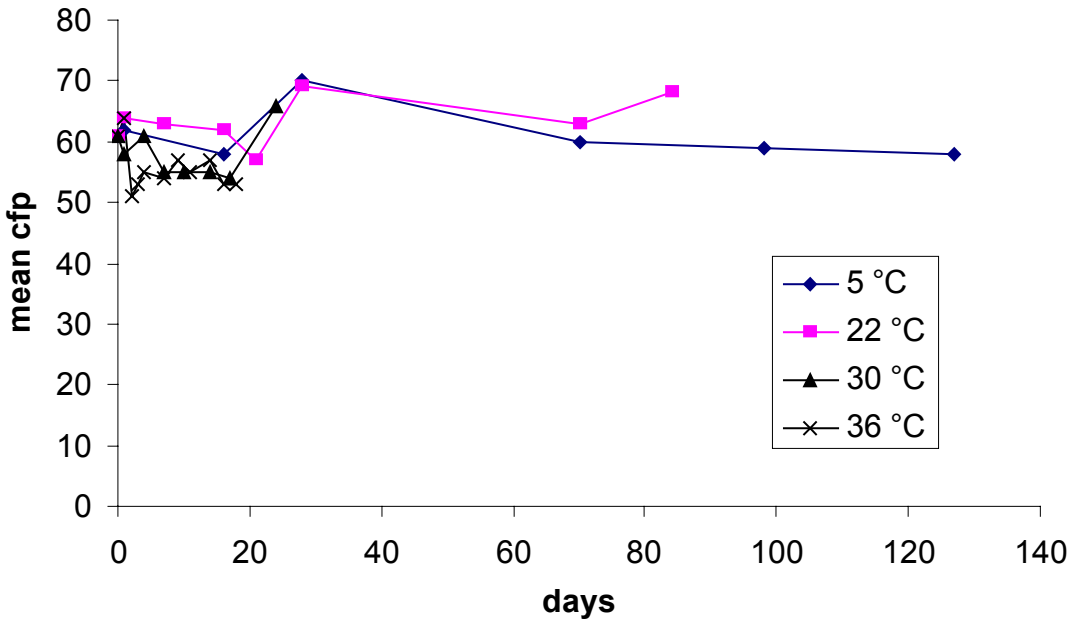

Figure 10 *Short-term stability study at elevated temperatures of batch of capsules LW34-24/07/01, containing Enterococcus faecium. Every measurement point gives mean number of 5 capsules enumerated in duplicate on YA, 22 °C (ISO 6222; Anonymous, 1999). Start study 10 June 2002.*

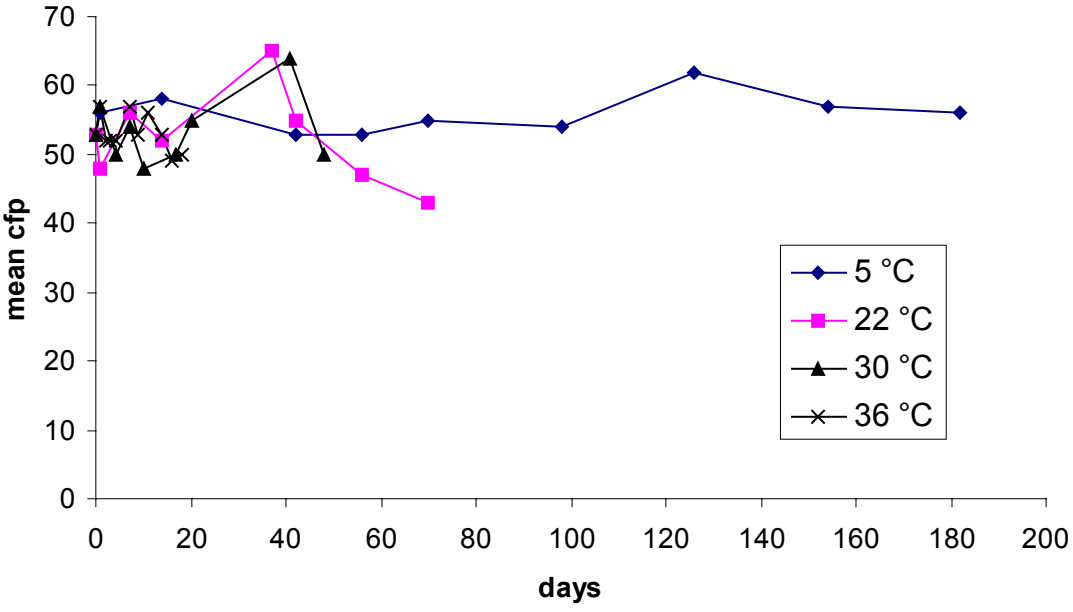

Figure 11 *Short-term stability study at elevated temperatures of batch of capsules LW34-24/07/01, containing Enterococcus faecium. Every measurement point gives mean number of 5 capsules enumerated in duplicate on YA, 36 °C (ISO 6222; Anonymous, 1999). Start study 3 December 2001.*

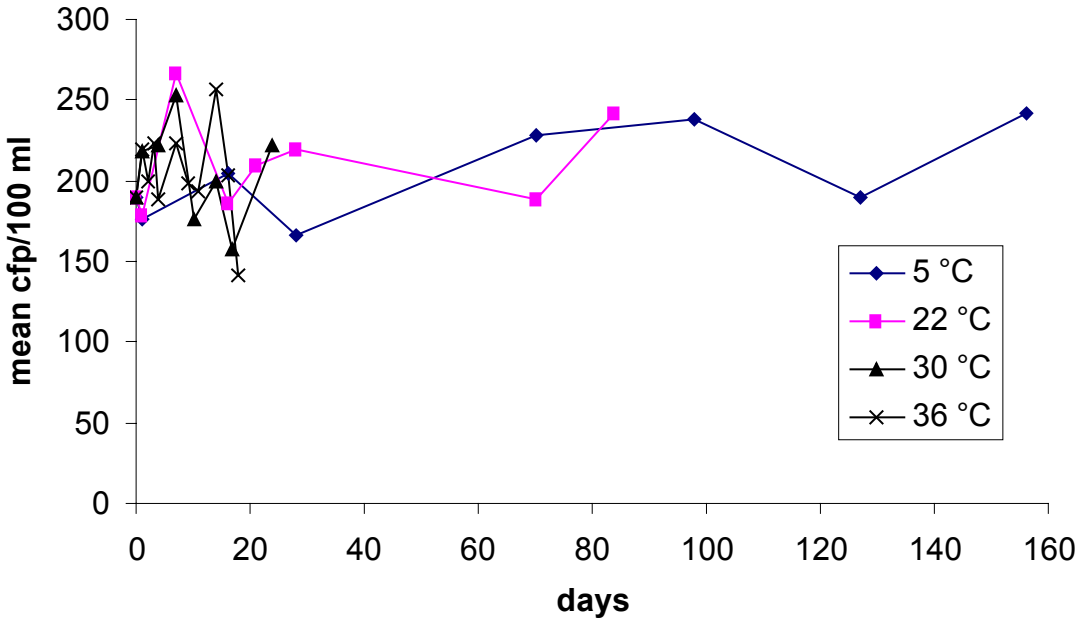

Figure 12 *Short-term stability study at elevated temperatures of batch of capsules LW34-24/07/01, containing Enterococcus faecium. Every measurement point gives mean number of 5 capsules enumerated in singular on microtitre plates (ISO 7899-1; Anonymous, 1998a). Start study 10 June 2002.*

3.3 *Clostridium perfringens* (D10; NCTC 13170)

The pre-set criteria for the batch of RMs containing *Clostridium perfringens* were:

- mean contamination level: between 250 and 750 cfp/capsule;
- T_1 not significantly from a χ^2-distribution with I (= the number of tested capsules) degrees of freedom;
- $T_2/ (I-1) \leq 2$;
- $T \leq 3$ (preferably ≤ 2).

In Table 4 the results of the test batch are given. As each capsule was reconstituted in 10 ml peptone saline solution (see Annex 5) and of each solution 1 ml was enumerated according to Annex 6 on TSC (ISO/WD 6461-2; Anonymous, 2001), the counted mean level was expected to lie between 25 and 75 cfp.
The test batch fulfilled the pre-set criteria so that the final batch was prepared. The capsules were homogeneously filled as s / $\bar{x}$ was 1.4 % ($\bar{x}$ = 0.292 g and s = 0.004 g).

The homogeneity of the final batch was tested on TSC (Anonymous, 2001). The results are given in Table 4.

The raw data of all homogeneity test results are given in Annex 7.
T_1 of the final batch showed a higher variation than expected for a Poisson distribution. This high variation within capsules was mainly caused by 3 capsules (no 1, 5 and 22, see raw data in Annex 7) out of the 30 tested capsules. As the mean level and the variation between capsules (T_2 /(I-1) as well as T) fulfilled the criteria, the batch was accepted for further use.

The long-term stability study started in November 2001 on TSC (ISO/WD 6461-2; Anonymous, 2001). Results are given in Figure 13. In this figure the individual (5) capsule results (duplicate means) are indicated as dots and a line is drawn through the mean result per measurement point. Also the results of the homogeneity test is indicated ('hom', 30 capsules). The results of the long-term stability studies showed stable results for the period measured (13 months).
Results of the short-term stability study at elevated temperatures are given in Figure 14. The batch of capsules containing *Clostridium perfringens* showed stable results at all tested storage temperatures for the period measured. As the materials were stable, no half-lifes were calculated (further details are given in Annex 4).
The raw data of the stability studies are given in Annex 8 (long-term) and Annex 9 (short-term).

Table 4 Results of homogeneity tests of test batch and final batch (LWL3501-24/10/01) of capsules containing Clostridium perfringens.

	I	J	Mean cfp[2]	T_1[3]	T_2/ (I-1)	T
Test batch on TSC[1]						
Tested October 2001	10	2	56.4	13.8	1.2	nt
Final batch on TSC[1]						
Tested November 2001	30	2	56.0	60.5	1.7	1.5

[1]: ISO/WD 6461-2: 2001, TSC: Tryptose Cycloserine agar;
I: number of tested capsules; J: number of tested replicates.
[2]: On TSC, 1 ml of the reconstituted capsule solution (10 ml) was enumerated. The mean number of cfp counted on the filters is given.
[3]: Upper limits of the χ^2 distribution: 18.3 (at I=10) and 43.8 (at I=30).
nt: not tested

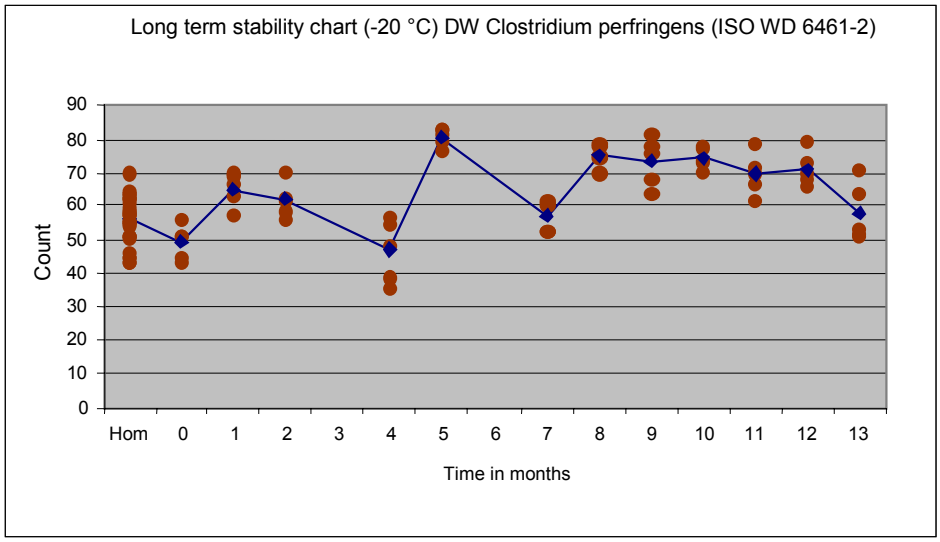

Figure 13 *Long-term stability study of batch of capsules LWL3501-24/10/01, containing Clostridium perfringens, stored at –20 °C. Every dot indicates the duplicate mean of individual capsule results being 30 capsules for the homogeneity study (hom) and 5 capsules for the stability study, enumerated in duplicate on TSC agar (ISO/WD 6461-2; Anonymous, 2001). Start study 7 November 2001.*

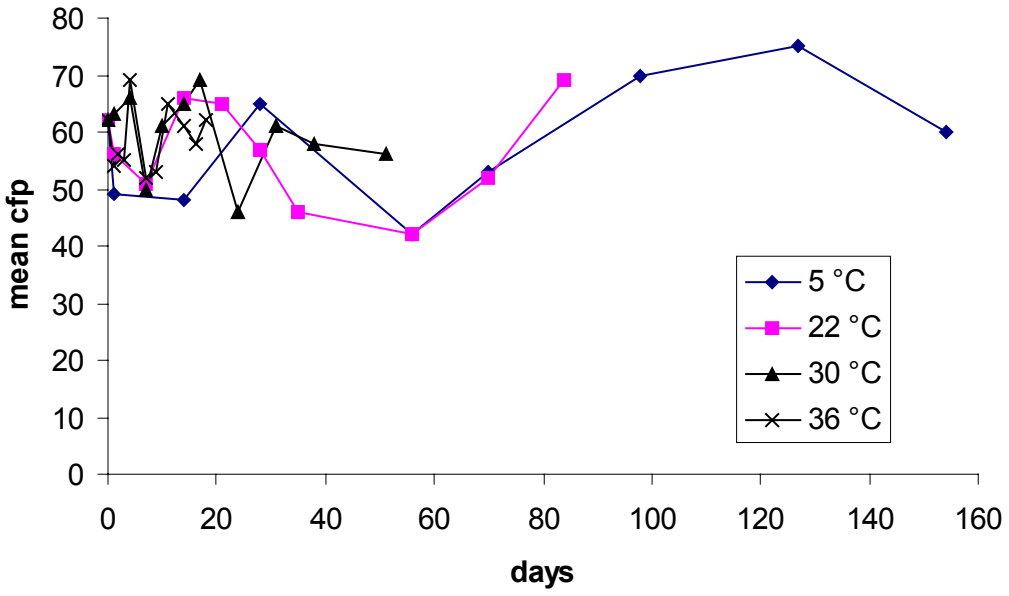

Figure 14 *Short-term stability study at elevated temperatures of batch of capsules LWL3501-24/10/01, containing Clostridium perfringens. Every measurement point gives mean number of 5 capsules enumerated in duplicate on TSC agar (ISO/WD 6461-2; Anonymous, 2001). Start study 14 January 2002*

3.4 *Pseudomonas aeruginosa* (CIP 82118)

The pre-set criteria for the batch of RMs containing *Pseudomonas aeruginosa* were:
- mean contamination level: between 250 and 750 cfp/capsule;
- T_1 not significantly from a χ^2-distribution with I (= the number of tested capsules) degrees of freedom;
- $T_2/ (I-1) \leq 2$.

In Table 5 the results of the test batch are given. As each capsule was reconstituted in 10 ml peptone saline solution (see Annex 5) and of each solution 1 ml was enumerated according to Annex 6 on CN-agar (prEN 12780; Anonymous, 1999), the counted mean level was expected to lie between 25 and 75 cfp.
The mean contamination level of the test batch was lower than expected. However, as the colonies of this strain were relatively large it would be an advantage to have a lower number of colonies on the filter. Therefore it was decided to prepare the final batch. The capsules were homogeneously filled as s / $\bar{x}$ was 2 % ($\bar{x}$ = 0.300 g and s = 0.007 g).
The homogeneity of the final batch was tested on CN-agar (Anonymous, 1999). The results are given in Table 5. Homogeneity was only tested for a Poisson distribution ($T_2/(I-1)$) and not for 'reproducibility' (T).
The raw data of all homogeneity test results are given in Annex 7.

Results of the stability study of the materials stored at –20 °C are given in Figure 15. The first two measurement points in this figure are results of the test batch, the others of the final batch. For all measurements 10 capsules were enumerated in duplicate, except for the final measurement (day = 208), where 5 capsules in duplicate were enumerated. The stability study at elevated temperatures was not performed as the RMs were not even stable when stored at –20 °C. No further analysis was applied on the stability test results, as it was obvious that the batch was not stable.
The raw data of the stability study are given in Annex 8.

Table 5 Results of homogeneity tests of test batch and final batch (LWL36-21/11/01) of capsules containing Pseudomonas aeruginosa.

	I	J	Mean cfp	T_1 [3]	T_2/ (I-1)
Test batch on CN-agar[1]					
Tested November 2001	10	2	14.8 [2]	13.7	0.6
Final batch on CN-agar[1]					
Tested December 2001	10	2	9.1 [2]	15.8	0.6

[1]: prEN 12780: 1999; I: number of tested capsules; J: number of tested replicates.
[2]: Of the capsules of the test batch, 1 ml of the reconstituted capsule solution (10 ml) was enumerated. Of the final batch 2 ml of the reconstituted capsule solution (10 ml) was enumerated. The mean number of cfp counted on the filters is given.
[3]: Upper limit of the χ^2-distribution: 18.3 (at I=10).

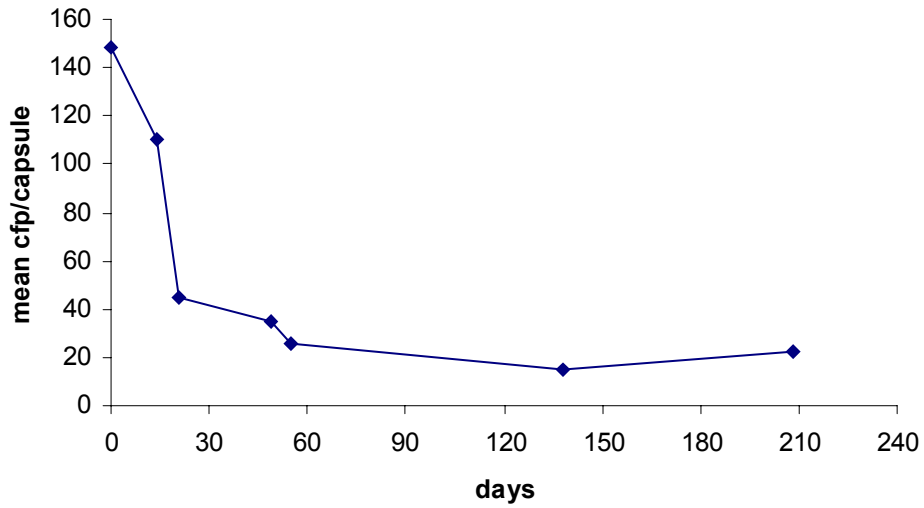

Figure 15 Stability study of batch of capsules LWL36-21/11/01, containing Pseudomonas aeruginosa, stored at –20 °C. Every measurement point gives mean number of 5-10 capsules enumerated in duplicate on CN-agar (prEN 12780; Anonymous, 1999). Start study 21 November 2001.

4. Discussion and conclusions

In earlier studies it has been shown that in general the highly contaminated milk powders needed a stabilisation period before they could be used for preparing stable RMs (Mooijman *et al*., 1992). The length of this period was depended on the test strain. For the preparation of the batches of capsule RMs in this report it was possible to use earlier prepared and stabilised hcmp's resulting in relatively stable RMs. However, this was not the case for the RMs containing *Pseudomonas aeruginosa*. For this material a new hcmp needed to be prepared. The period between preparation of the hcmp and mixing and filling of the capsules was too short to stabilise the hcmp. This was clearly shown in the stability test results of this batch. As time was too short to prepare a new batch of hcmp and/or to make a new mixture before the feasibility certification studies, it was decided not to use this batch of *Pseudomonas aeruginosa* capsules for the feasibility certification studies.

The batch of capsule RMs containing *Escherichia coli* fulfilled the aim for the mean contamination level on the intended methods (51.1 cfp on LTTC and 530 cfp/ 100 ml on microtitre plates). However, the homogeneity results of the batch were somewhat variable. The test batch was tested on LSA and fulfilled the pre-set criteria. The final batch was at first instance also tested on LSA and these results were also close to the pre-set criteria (homogeneity only tested for Poisson distribution). The analyses on LSA of the test batch and of the final batch were performed soon after the preparation of the batch. By that time, LTTC and microtitre plates were not yet available. It was decided to accept the batch on account of the results found on LSA. Later studies performed on LTTC and on the microtitre plates showed good results for the mean contamination level, but elevated variation between capsules when tested for a Poisson distribution (T_2). However, when homogeneity was tested for 'reproducibility' (T), the results fulfilled the most strict criterion for this test (≤ 2). These results show (again) the dependence of microbiological results on the method used. Furthermore it shows that T_2 (testing for a Poisson distribution) is not always the best choice for testing homogeneity of a batch of RMs.

The stability of the *E. coli* capsules was good when stored at –20 °C. However, at elevated temperatures the material was less stable. No large differences were found between the tested media (LTTC and microtitre plates). Both showed the same trend and similar half-life values. Earlier studies on stability of *E. coli* capsules at elevated temperatures showed similar results (Mooijman *et al*., 1996). By then it was decided that this type of (C)RM (containing *E. coli*) needed to be cooled during transport. However, at that time (study was performed in 1994) the rules for sending microbiological materials as dangerous goods were less strict than nowadays. The packages for dangerous goods do not offer the possibility to include cooling elements, thus making it more complicated to cool the packages during transport. The only remaining way is to order the courier service to keep the packages cool as much as possible.

However, it will depend on the courier and/or the local authorities whether this will work well.

The batch of capsules containing *Enterococcus faecium* fulfilled all pre-set criteria for the mean contamination level as well as for the homogeneity for all tested media. Also the stability of this batch of RMs was very good at all tested temperatures with all tested media. Even storage of the RMs at 36 °C caused almost no effect on the mean contamination level. This RM containing *E. faecium* showed its functionality to its possible use in four different methods and to the possibility of mailing it without cooling.

The RMs containing *Clostridium perfringens* fulfilled the criteria for the mean contamination level and for the variation between capsules. The variation within capsules (T_1) of the final batch was higher than expected for a Poisson distribution. High variation between duplicate counts is in most cases a technical problem. In this case, 3 capsules out of the 30 tested capsules caused the high value of T_1. As the other criteria were still fulfilled, the batch was accepted for further use.
Like for the batch of RMs containing *E. faecium,* this batch containing *Cl. perfringens* was stable at all tested storage temperatures. This good stability can be explained by the fact that this type of RM contained a spore suspension of *Cl. perfringens*.

Summarising it can be said that the batches of capsule RMs, except the batch containing *Pseudomonas aeruginosa,* are of good quality and can be used in the feasibility certification studies.

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Annex 1 Biochemical determination results of the test strains

***Escherichia coli* (WR1; NCTC 13167)**
Strain *E. coli* (WR1) was originally isolated from water. The reference number WR1 is a number used at the Microbiological Laboratory for Health Protection of the RIVM. The strain was extensively tested in the first certification study. Results of this determination are summarised in the report on the certification study of CRM594 (Annex A of Mooijman *et al.,* 1996). By then the strain was biochemical confirmed as *Escherichia coli* by an 'internal' (RIVM) laboratory and by an external laboratory (Institute Pasteur in Paris).
In November 2000 a (limited) determination of the strain was repeated at the Laboratory of Infectious Disease Diagnostics and Screening of the RIVM to check the quality of the strain. Results of this latter biochemical determination are given below. The strain was again confirmed being *Escherichia coli*.

Microscopic observations	Gram – rods
Motility	+
Growth:	
Aerobic	+
Anaerobic	+
at 37 °C	+
on Mac Conkey	+
Malonate	-
Citrate	-
Gas from Glucose	+
Fermentation of:	
Adonitol	-
Arabinose	+
Dulcitol	+
Fructose	+
Galactose	+
Glucose	+
Lactose	+
Maltose	+
Mannitol	+
Mannose	+
Melibiose	+
Melezitose	-
Raffinose	-
Rhamnose	+
Sorbitol	+
Sucrose	-
Trehalose	+
Xylose	+
Decarboxylation of:	
Lysine (LDC)	-
Ornithine (ODC)	+
Dehydrolysation of Arginine	±
Hydrolysis of Esculine	-
Production of:	
Acetoin (VP)	-
Catalase	+
ß-Galactosidase	+
H_2S	-
Indole	+
Oxidase	-
Urease	-
Reduction of Nitrate	+
Methyl red test	+
Haemolysis (α/β)	-

***Enterococcus faecium* (WR63; NCTC 13169)**

Strain *E. faecium* (WR63) was originally isolated from water. The reference number WR63 is a number used at the Microbiological Laboratory for Health Protection of the RIVM. The strain was extensively tested in an earlier certification study. Results of this determination are summarised in the report of the certification study of CRM06 (Annex A of Mooijman *et al.,* 1999). By then the strain was biochemical confirmed as *Enterococcus faecium* by an 'internal' (RIVM) laboratory and by an external laboratory (Faculty of Veterinary Medicine of the University of Gent).
In November 2000 a (limited) determination of the strain was repeated at the Laboratory of Infectious Disease Diagnostics and Screening of the RIVM to check the quality of the strain. Results of this latter biochemical determination are given below. The strain was again confirmed being *Enterococcus faecium*.

Microscopic observations	Gram + cocci
Motility	-
Growth:	
Aerobic	+
Anaerobic	+
at 37 °C	+
Gas from Glucose	-
Fermentation of:	
Arabinose	+
Glucose	+
Glycogen	-
Inulin	-
Lactose	+
Mannitol	+
Raffinose	-
Ribose	+
Sorbitol	-
Starch	-
Trehalose	+
Dehydrolysation of Arginine	+
Hydrolysis of:	
Esculine	+
Hippurate	+
Production of:	
Acetoin (VP)	+
Alkaline phosphatase	-
Catalase	-
α-Galactosidase	-
ß-Galactosidase	+
β-Glucuronidase	-
Leucine arylamidase	+
Oxidase	-
Pyrrolidonyl arylamidase	+
Haemolysis (α/β)	-

***Clostridium perfringens* (D10; NCTC 13170)**

Strain *Clostridium perfringens* (D10) was originally isolated from a patient and from food. The reference number D10 is a number used at the Microbiological Laboratory for Health Protection of the RIVM. Determination of the strain was performed in 1993, 1994 and in November 2000 at the Laboratory of Infectious Disease Diagnostics and Screening of the RIVM. The three determinations gave similar results. Results of the biochemical determination are given below. The strain was confirmed being *Clostridium perfringens*.

Microscopic observations	Gram + rods
Motility	-
Growth:	
Aerobic	-
Anaerobic	+
at 37 °C	+
on Mac Conkey	±
Malonate	-
Citrate	-
Gas from Glucose	+
Fermentation of:	
Adonitol	-
Arabinose	-
Dulcitol	-
Fructose	+
Galactose	+
Glucose	+
Inositol	+
Lactose	+
Maltose	+
Mannitol	-
Mannose	+
Melibiose	-
Melezitose	-
Raffinose	±
Rhamnose	-
Salicine	-
Sorbitol	-
Sucrose	+
Trehalose	±
Xylose	-
Decarboxylation of:	
Lysine (LDC)	-
Ornithine (ODC)	-
Dehydrolysation of Arginine	±
Hydrolysis of Esculine	-
Production of:	
Acetoin (VP)	-
Amylase	+
Caseinase	-
Catalase	-
Coagulase	+
ß-Galactosidase	+
Gelatinase	+
H_2S	+
Indole	-
Lecithinase	+
Lipase	-
Oxidase	-
Urease	-
Reduction of Nitrate	+
Methyl red test	±
Haemolysis (α/β)	β

***Pseudomonas aeruginosa* (CIP 82118)**

Strain *Pseudomonas aeruginosa* (CIP82118) was obtained from the culture collection of Institute Pasteur in Lille (France). In 2001 biochemical determination of the strain was carried out before spray-drying (July 2001) and after drying (December 2001) at the Laboratory of Infectious Disease Diagnostics and Screening of the RIVM. Both determinations confirmed the strain being *Pseudomonas aeruginosa*. Results of the biochemical determination are given below.

Microscopic observations	Gram - rods
Motility	+
Growth:	
Aerobic	+
Anaerobic	-
at 37 °C	+
at 42 °C	+
on Mac Conkey	+
Malonate	+
Citrate	+
Cetrimide	+
Fermentation of:	
Fructose	+
Galactose	+
Glucose	+
Lactose	-
Maltose	-
Mannitol	+
Mannose	+
Rhamnose	-
Sucrose	-
Xylose	+
Formation of pigment	+
Decarboxylation of:	
Lysine (LDC)	-
Ornithine (ODC)	-
Dehydrolysation of Arginine	+
Hydrolysis of Esculine	-
Production of:	
Acetoin (VP)	-
Amylase	-
Caseinase	+
Catalase	+
ß-Galactosidase	-
H_2S	-
Indole	-
Lecithinase	-
Lipase	+
Oxidase	+
Urease	+
Reduction of Nitrate	+
Methyl red test	-
Haemolysis (α/β)	-

Annex 2 Part of report on WP1: Objectives specification

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1. Introduction

The MICROCRM project started on 1 February 2001. On 4-6 March 2001 the first meeting was organised in Lille (France) with participating laboratories and partners of the project. Here the project was introduced to the participants. The aim of the MICROCRM project (pilot feasibility study) is to determine the conditions that are necessary to produce and certify key water microbiological reference materials (RMs) that will support EU Water legislations (Drinking Water and Bathing Water Directives). The minutes of the meeting are given in Annex 1.

2. Selection of micro-organisms, concentration levels and methods

During the first meeting with participants, agreements were made on the chosen strains, concentration level in the final analytical portions and on the analytical methods. As the final certified reference materials (CRMs) should support EU water legislations it was decided to select the methods as indicated in these directives. However, it needs to be remarked that the new Bathing Water (BW) Directive is still under discussion. At the moment of the meeting in Lille (March 2001) two microbiological parameters and their relevant methods have been suggested for the new BW Directive. Also a suggestion is indicated in this draft BW Directive for the maximum allowable number to be found for these parameters in bathing waters. These figures were used as basis for determining the target values for the CRMs. However, the suggested method for intestinal enterococci has a detection limit of 15 colony forming particles per 100 ml (cfp/ 100 ml). Meaning that this method will give higher variation in results when analysing samples with low counts than when analysing samples with relatively high counts. Thus using this method for analysing a CRM with a target value of 50 cfp/100 ml intestinal enterococci (the draft value of the BW Directive) might give large variation. It was therefore decided to apply a feasibility certification study for a CRM containing 200 cfp/100 ml intestinal enterococci. In the laboratories of the partners these materials will also be diluted (after reconstitution) to a level of *ca* 50 cfp/100ml to check the influence of dilution as well as the influence of the relatively low contamination level on the variation of results obtained with the specified method.
Finally, the method indicated in the Drinking Water (DW) Directive for the enumeration of *Clostridium perfringens* has been thoroughly discussed in ISO-meetings (ISO/TC147/SC4/WG5). Some delegates in these ISO-meetings also have tested several methods for the enumeration of *Clostridium perfringens.* From the results of these studies, Working Group 5 (WG5) of ISO/TC147/SC4 decided to prepare an ISO method based on another medium than the one indicated in the DW Directive. The participants and partners of the MICROCRM project therefore decided during the meeting to follow the ISO(/WD) method instead of the one stated in the DW Directive.

The list of micro-organisms, target levels and methods are summarised in Table 1.

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 / 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

The partners agreed that each partner could choose its own relevant strain for the preparation of their own batches of RMs. The criteria for each chosen strain are:

- Representative strain, preferably from environment;
- Strain traceable to a culture collection.

The partners discussed whether it would be necessary to make duplicate counts of each RM or not. It was agreed that the choice on making duplicates would depend on the application of each RM. Lenticules (RMs of PHLS) are designed to use as a whole without making duplicates. For some applications, like presence/absence tests the capsules (RMs of RIVM) can be used as a whole. However, in case of enumeration methods it is technically preferred to take subsamples out of the reconstituted capsule solution. Pastilles (RMs of IPL) can be used in both ways (for making duplicates and enumeration in singular). It was decided that for the certification feasibility studies the number of 'Units' (individual lenticules, pastilles or capsules) of each RM as indicated in Table 2 will be tested.

Table 2 Number of 'Units' of each 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

3. Homogeneity test

It was decided that the homogeneity test designed at RIVM would be used to determine the homogeneity of the batches of RMs of the three partners. For the enumeration of the RMs the methods of Table 1 will be used.

For the homogeneity test the following calculations are made (Heisterkamp *et al*., 1993).

In the case where duplicate counts of the individual 'Units' of the RMs are made (see table 3) the variation between duplicates is calculated with the so-called T_1 statistic.

$$T_1 = \sum_i \sum_j [(z_{ij} - z_{i+}/J)^2 / (z_{i+}/J)]$$

Where z_{ij} is the number of cfp per analytical portion (j) of unit i.

$z_{i+} = \sum_{j=1}^{2} z_{ij}$ is the sum of numbers of cfp in both duplicates of unit i.

J is the number of analytical portions (replicas) per unit. In this study J is always 2.

Variation between different 'units' from one sample from a batch of a particular RM is calculated with the so-called T_2 statistic.

$$T_2 = \sum_i [(z_{i+} - z_{++}/I)^2 / (z_{++}/I)]$$

Where $z_{++} = \sum_i (\sum_j z_{ij})$ is the sum of all cfp in all the I units of one sample.

I is the total number of units in a sample.

In the case of a Poisson distribution, T_1 and T_2 follow approximately a χ^2-distribution with I and (I-1) degrees of freedom respectively in this case. Also in this case the expected values of T_1 and T_2 are approximately the same as the number of degrees of freedom. Hence, T_1/ I and T_2/ (I-1) are expected to be close to one.

In earlier work it was shown that it is possible to find a T_1 value in practice that does closely follow a χ^2- distribution. However, the T_2 value is in most cases larger than a true χ^2-distribution would suggest, indicating dispersion greater than that associated with the Poisson distribution.

To measure the homogeneity of a sample of units, T_2/ (I-1) is calculated. If $T_2/(I-1) \leq 2$, the homogeneity of the sample, and hence the batch, is probably acceptable.

To determine the homogeneity of a batch of 1000 RMs (lenticules/pastilles/capsules) immediately after production, a minimum sample of 30 'units' should be analysed (either in singular or in duplicate, depending on the type of RM, see Table 3). The 'units' to be tested should be taken at random. If the sample is taken sequentially during the production process, a record should be kept of when during the process, these samples are taken, in order to check for changes to the cfp count over time.

To perform the T_1 and T_2 calculations an Excel file has been designed at the RIVM/MGB. RIVM/MGB will make this file available to the partners of the project.

4. Stability studies

To check the stability of the RMs two studies will be necessary:
a. Long-term stability studies at storage temperature;
b. Short-term stability studies at elevated temperatures;
c. Transport study.

a. Long-term stability studies at storage temperature
For checking long-term stability the following study is designed:

Immediate after production of each batch of RMs all 'units' are packed in their relevant packages and stored at (-20 ± 5) °C. Every month 5 'units' of each RM will be analysed, using the methods as indicated in Table 1 (either in singular or in duplicate, depending on the type of RM, see table 3).

Note: Lenticules have always been stored at (-25 ± 5) °C. It will be checked by PHLS whether these RMs are sufficiently stable at (-20 ± 5) °C as well.

Table 3 Numbers of units of RMs to be sampled in Homogeneity and Stability testing

RM Type	Homogeneity Testing	Stability Testing 100 ml samples	Stability Testing 1 ml samples
Lenticules	30 in singular	5 in singular	5 in duplicate
Pastilles	30 in singular	5 in singular	5 in duplicate
Capsules	30 in duplicate	5 in duplicate	5 in duplicate

b. Short-term stability studies at elevated temperatures
The rationale of the following study is to determine the useful stable lifetime of the RMs under different storage temperatures. The study will check units of each RM under a changing sequence, there being twice as many checks in the early stages. Table 4 determines the 'rate' of the checking for all the temperatures. The laboratories will record the cumulative time to each check, to the nearest hour, (time being from removal from storage at (-20 ± 5) °C).

Plan
Soon after production of the batches of RMs, 205 'units' of each batch of RM are to be taken out of (-20 ± 5) °C and stored at higher temperatures. 50 units of each RM will be stored at each of the four higher temperatures shown in table 4. The remaining 5 units of each RM should be tested immediately.

The units stored at the higher temperatures will be checked according to the schedule in Table 4 using the relevant methods (5 'units' per check). This will start at the faster rate but will slow after five checks if no fall in mean count has been seen by that time. By this we mean that if the mean cfp count does not fall below 1.645 standard errors of the mean (see

formula below) under the initial mean cfp count (established by the homogeneity study - see section 3 above) within the first 5 checks, the rate of checking will then slow. In any event, continue checking until either:

1) The number of units of the RM runs out.
2) The cfp count reaches zero.

This procedure will enable statistical models of RM lifetime, with good estimates of the limit of useful storage times, to be derived for many temperatures, not just the ones studied directly.

Data collection forms will be designed for collection of all data related to these studies prior to the commencement of the tests.

Formula for assessing fall in mean count of cfp

If *C* is the (arithmetic) mean count (established under the Homogeneity study)
S is the standard deviation (between the 30 counts recorded)

Then a fall exceeds 1.645 standard errors of the mean if the count (of 5 units) falls below

$$C - 1.645\sqrt{S^2/5}$$

Table 4 Sampling rates for different storage temperatures

	Storage Temperatures			
Check #	**(36 ± 2) ^{0}C**	**(30 ± 2) ^{0}C**	**(22 ± 2) ^{0}C**	**(5 ± 3) ^{0}C**
1	The first 5 units immediately after removal from the store at **–20 ^{0}C**			
2-6	Daily	Twice a week	Once a week	Once every two weeks
	If there is an observed fall in cfp count by this stage (greater than 1.645 standard errors of the mean) then:			
7-11	Daily	Twice a week	Once a week	Once every two weeks
	If there is NOT an observed fall in cfp count by this stage (none greater than 1.645 standard errors of the mean) then:			
7-11	Every two days	Once a week	Every two weeks	Every four weeks

c. Transport study

The main influence on the stability of the RMs during transport is temperature. The results of the long-term stability studies and of the short-term stability studies will be used to determine whether performing an extra transport study would be necessary. The design of the transport study will be worked out per batch of RM if necessary.

5. Packaging of the RMs

Depending on the type of RM the packaging can be ‘singular’ packaging (1 ‘unit’ per package material) or ‘multiple’ packaging (several ‘units’ per package material). As the lenticules are relatively fragile, singular packaging is preferred to be sure that complete lenticules are analysed. In case of the capsules and the pastilles multiple packaging can be used as well.

6. Batch size

To have sufficient ‘units’ of each RM to perform all analyses, including the training session and the certification feasibility studies, a batch size of *ca* 1000 ‘units’ for each type of RM is necessary.

References

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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. Second edition, 1998. International Organization for Standardization, Geneva, Switzerland.

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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. First edition, 1998. International Organization for Standardization, Geneva, Switzerland.

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Heisterkamp S.H., Hoekstra J.A., van Strijp-Lockefeer N.G.W.M., Havelaar A.H., Mooijman K.A., in 't Veld P.H. and Notermans S.H.W. (1993) Statistical analysis of certification trials for microbiological reference materials. EUR 15008 EN. Commission of European Communities, Community Bureau of Reference, Brussels, Luxembourg.

Annex 3 Homogeneity studies for microbiological reference materials

Introduction and background
Homogeneity studies are carried out for microbiological reference materials in order to establish the between units (e.g. capsules, pastilles, or lenticules) variation.

Variation may arise as a consequence of:
- heterogeneities introduced during the manufacturing process,
- variation in handling between manufacturing and use, and
- variation in testing procedures (quality of media, experience in counting typical colonies etc).

Homogeneity studies generally aim at establishing the first source of variation. Therefore such studies are generally carried out within a single, experienced, laboratory only.

Methods
There generally exists a minimum level of heterogeneity determined by the target value (or actually the true value) of the reference material and the Poisson distribution. Let μ be the mean number of cfp (colony forming particles) per unit, then the (Poisson) distribution of cfp in homogeneously manufactured units is

$$Pr(cfp = k) = \mu^k \exp(-\mu) /k!$$

where k! (k faculty) denotes k(k-1)(k-2)(k-3)…3.2.1. Pr (cfp = k) denotes the probability of obtaining a count of exactly k cfp.
The expected value ("theoretical mean") of k is denoted by μ. In many studies the parameter is the one of interest and needs to be estimated.

This (Poisson) distribution has the property that the standard deviation equals the square root of the mean (μ). The coefficient of variation thus decreases with the mean. This distribution constitutes the null hypothesis. The alternative hypothesis is that the variance is larger than the mean. Such a larger variance is commonly known as overdispersion or extra-Poisson variation, and is generally caused by non-homogeneous material. Non-homogeneities may be inevitable during the manufacturing process (e.g. due to "clumping" of cfp). These would not necessarily disqualify materials from use as reference materials, provided they are properly explored and documented.

1. Test for Poisson distribution

Tests for the Poisson distribution are generally based on the principle that the mean equals the variance. Well known is the T_2 statistic. Let $k_1, k_2, \ldots, k_I$ be counts obtained from I randomly chosen units of the reference material. Then,

$$T_2 = \Sigma (k_j - k_+/I)^2/(k_+/I)$$

(where k_+ denotes Σk_j , i.e. the sum over k_j), has approximately a chi-square distribution with (I-1) degrees of freedom, if the k_j have a Poisson distribution.

Note, that if a random variable X has a Chi-square (χ^2) distribution, with (I-1) df (degrees of freedom), then its expectation E(X), and variance $\sigma^2(X)$ are

$$E(X) = (I-1),\ \sigma^2(X) = 2(I-1)$$

A complication arises with the microtitre 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 we can analyse these data 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.

Required sample size for testing for homogeneity using T_2.
Consider the alternative hypothesis (i.e. a distribution with extra-Poisson variation) with mean, variance and variance-of-variance.

$$E(k) = \mu,$$

$$\sigma^2(k) = f\mu \ (f>1),$$

$$\sigma^2(T_2) = 2(1+2(f-1))(I-1); \quad (f>1)$$

That is T_2 has a non-central chi-square distribution under the alternative hypothesis of heterogeneity. The parameter f is a parameter that determines the extra-Poisson variation in

the distribution of the counts. It is the large sample mean of $T_2/(I-1)$. If f =1 we have the Poisson distribution (i.e. the null hypothesis of homogeneity).
Under this alternative hypothesis, the expected value of T_2 is approximately f(I-1). As for moderately large degrees of freedom (say I > = 5) the Chi-square distribution resembles the normal distribution, sample size calculations can be (approximately) based on the normal distribution.

The probability that T_2 exceeds $(I-1) + 2\sqrt{(2(I-1))}$, i.e. the approximate critical value (significance level 0.05) of the Chi-square distribution, equals (approximately),

$$Pr(T_2 > (I-1) + 2\sqrt{(2(I-1))}\ ) = \Phi(\{2\sqrt{(2(I-1))}\ -\ (f-1)(I-1)\}/\ \{\sqrt{(2(I-1)(1+2(f-1))}\})$$

Where $\Phi(\ )$ denotes the cumulative standard normal distribution.

For a power of 0.80 we find that the number of observations (I) should be approximately

$$I = 1+ (2.8 + 0.84\sqrt{(2(1+2(f-1)))}\ )^2/(f-1)^2$$

Thus for f=2 (dispersion is twice the Poisson dispersion), 25 units should be adequate.

2. Test for reproducibility

Another method to report the homogeneity/heterogeneity (all sources of heterogeneity combined) is by methods related to classical reproducibility statistics. This method does not make explicit reference to the Poisson distribution.
Let x_1 and x_2 ($x_1 <= x_2$) be two (cfp) counts obtained from two randomly chosen units of a reference material, we then define T by requiring that

$$Pr(x_2 /x_1 < T) = 0.95$$

Estimation of T.

Our objective is to estimate T. T is defined as 'the number below which the ratio (max/min) of two randomly chosen observations count1 and count2 will be with probability 0.95'.
To avoid problems with zeroes it is better to work with x_1= count1+1, x_2 = count2+1.
The difference between the (natural) logarithms of the ratio of the two counts should thus be less than log(T) with probability 0.95.

For this difference we have

$$\sigma^2(\log(x_1) - \log(x_2))\} = 2\sigma^2(\log(x))$$

where σ^2 denotes the variance (the squared standard deviation).

From this, we easily find that T can be estimated by

$$T = \exp(1.96*\sqrt{2}*sd(\log(x))) = \exp(2.8*sd(\log(x)))$$

Where sd denotes the sample standard deviation of log(x).

Sample size
Objective: Suppose we require that the uncertainty in T is at most 30%, that is uncertainty in sd(log(x)) at most 30%.

The sample variance of log(x) is approximately distributed as $\sigma^2\chi^2_{I-1}/(I-1)$, where χ^2_{I-1} denotes a chi-square distribution with I-1 degrees of freedom, and σ^2 (here) denotes the "true" variance of log(x).
This has expected value σ^2, and variance $2\sigma^4/(I-1)$. Thus the relative error (coefficient of variation) of sd(log(x)) = $1.4/\sqrt{(I-1)}$. If we want this to be less than 0.3, we must make I-1 = 20 .

In essence, we make requirements about the coefficient of variation (CV) of T.

For various values of the required CV, we obtain.

CV	sample size
33%	18
30%	22
25%	32
20%	50
10%	200

In other words we reduce the CV of T by increasing the sample size.

Annex 4 Stability studies of microbiological reference materials

Introduction
The stability of the batches of RMs was tested in two ways:
a. Long-term stability studies at storage temperature (-20 C);
b. Short-term stability studies at elevated temperatures.
The design of both studies is given in Annex 2. Here the statistical analysis is summarised.

Estimation of half lifes
'Half lives' have been estimated by fitting a Weibull distribution model to the death rate of the micro-organisms. The 'Half life' is an estimate of the time for half of the organisms to die. This value can be estimated from the reliability curve associated with the Weibull distribution. The Weibull is named after its Swedish inventor and is well described in his original paper Weibull W (1951).

The distribution is useful for modelling lifetime distributions.

The *Weibull model* has 3 parameters, which are:

α - The scale parameter, which controls the amount of spreading from, left to right evident in the graph of the probability density function (pdf).

β - The shape parameter that determines the basic form of the pdf

γ - The location parameter, which establishes the position of the start of the left end of the distribution, the point on the x-axis at which the pdf begins.

The probability density function for the Weibull distribution is given by:

$$f(x) = \frac{\beta}{\alpha}\left(\frac{x-\gamma}{\alpha}\right)^{\beta-1} e^{-\left(\frac{x-\gamma}{\alpha}\right)^{\beta}}$$

where x must be >= γ, and is sometimes denoted t (for time).

The cumulative distribution function (cdf) is:

$$F(x) = \quad 1 - e^{-\left(\frac{x-\gamma}{\alpha}\right)^{\beta}}$$

All the functions used to describe the properties of the process or products being modelled are derived from these basic functions and the parameters that determine them. F(x) or F(t) is the failure rate function that describes the cumulative proportion of failed items at any point in time. The reliability function R(t) = 1- F(t) represents the reliability rate (or survival) function and f(t)/R(t) produces the hazard function that describes the instantaneous probability of failure for a product at any point in time.

These represent the most useful of the functions in practice. Several modern statistical computer packages have Weibull distribution outputs but these often require user input of the distribution parameters, for example Minitab version 12 offers no way of finding the parameters if they are unknown.

An example of the reliability curve from the Weibull spreadsheet is shown in Figure 4.1. To estimate the half-life the time in days from the x-axis is found when the proportion survived on the y-axis is 0.50.

Reliability R(t) = 1 – F(t)

Where F(t) is the cumulative distribution function and is given by:

$$F(t) = 1 - e^{-\left(\frac{t-\gamma}{\alpha}\right)^{\beta}}$$

$$\text{So } R(t) = 1 - \left(1 - e^{-\left(\frac{t-\gamma}{\alpha}\right)^{\beta}}\right)$$

As the plots have already been adjusted for gamma γ the equation reduces to

$$R(t) = e^{-\left(\frac{t}{\alpha}\right)^{\beta}}$$

For a survival rate of 0.50, i.e. when half of the organisms have died (The estimated half life), R(t)=0.50.

$$0.50 = e^{-\left(\frac{t}{\alpha}\right)^{\beta}}$$

$$\ln(0.5) = -\left(\frac{t}{\alpha}\right)^{\beta}$$

$$[-\ln(0.5)]^{\frac{1}{\beta}} = \frac{t}{\alpha}$$

$$\alpha[-\ln(0.5)]^{\frac{1}{\beta}} = t$$

The value t gives the estimated half-life.

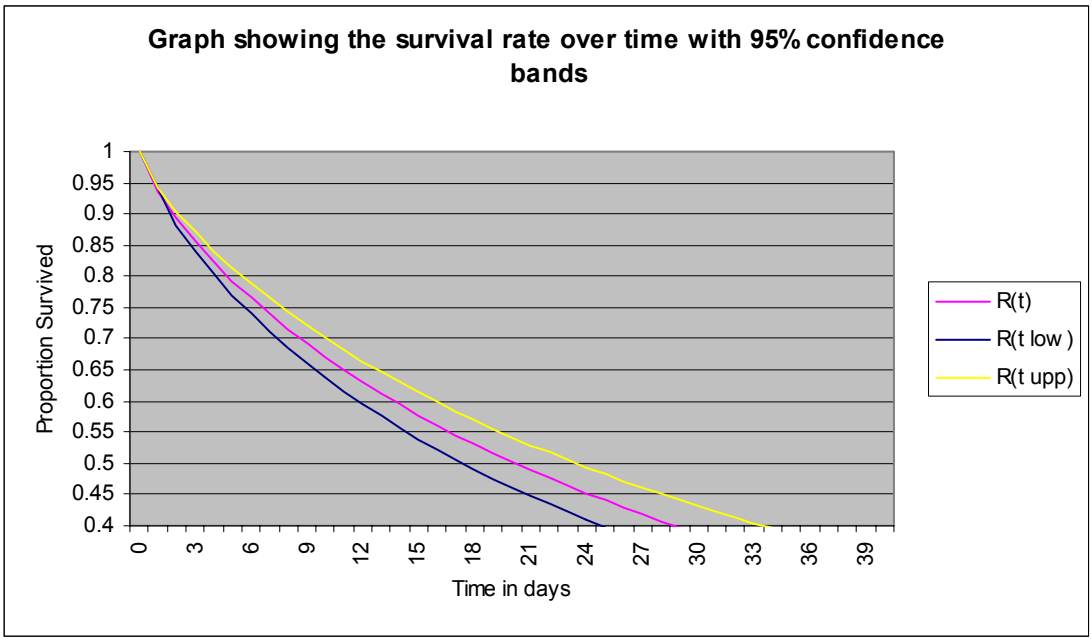

Figure 4.1 Reliability curve for the Weibull distribution

Long and short term variance components

The total variance observed in each stability study is broken down further into short and long-term components. The short-term variability is the within block variance. In this case the blocks are days, so the short-term variance is the average of the variances between the units of one batch of RMs found on each day.

The long-term variability is the between block variation, which is the variance between the average of each block – short term variability/number of observations in each block.

Note: The short-term variance of the long-term stability study has no connection with the short-term stability study. The same applies to the long-term variance of the short-term stability study, which has no connection with the long-term stability study.

Total variance = Long term variance + short term Variance

= Within block variation + (Between block variation – $\underline{\frac{\text{Within block variation}}{N}}$)

$$= \frac{\sum_{i=1}^{n} \text{var}(x_i)}{n} + \left[\frac{\sum_{i=1}^{n} (\bar{x}_i - \bar{\bar{x}})^2}{n} - \frac{\frac{\sum_{i=1}^{n} \text{var}(x_i)}{n}}{N} \right]$$

Where N = the number of observations per block

n = the number of blocks

$\bar{\bar{x}}$ is the average of the averages for each block (day)

A good indication of whether the RMs are stable is to compare the overall average counts from the stability study with the mean from the homogeneity study. If the mean count observed in the homogeneity study is similar to those observed for the long-term stability study, then it is likely that the material is stable.

Also the variance from the stability study gives an indication of the stability of the RMs. If most of the variance is from the short-term component then stability is implied. The short-term variation is the average of the variances for each block (day). For each day the variance is calculated for the 5 units of one batch of RMs. An average of these daily variances is then calculated. This gives the short-term variance.
If over the long term the variance is small, then the mean cfp values will be quite similar over this time period. This shows stability, as the changes are quite small, i.e. the material is stable (the cfp value remains quite constant)
If there is a lot of variation in the long-term variance component instability is expected, as largely differing values over the long term show the material is not stable.

Criteria for deciding if the reference material is stable
The mean obtained from the homogeneity study is used to assess the stability of the different organisms. It is the stability of the mean count that is of interest. It should be remembered that there is both long and short-term variability around the mean count. It is of interest whether the mean count remains stable given this variability.

To assess the stability of the mean of the long-term stability study, a 99% confidence band is fitted around the mean from the homogeneity study. The between block variation is used to get the confidence band. This is the variation observed around the averages of each block of 5 counts. So the averages are found for each month, then the variance of these averages is found. From this the standard deviation can easily be found which can then be used to set up the confidence band.

To establish a 99% confidence band the following formula is used:

$$C = \bar{x} \pm 2.5758 \times sd$$

Where $\bar{x}$ is the homogeneity study mean
and sd is the standard deviation from the average monthly counts

Results
Results of the variance components are given in Table 4.1 for the long-term stability results of the capsule RMs and in Table 4.2 for the short-term stability results of the capsule RMs.

Table 4.1 Long-term stability results for capsules

Micro organism	Stable	Estimated half life	Stability				Homogeneity	
			Short-term variance	Long-term variance	Total variance	Mean cfp	Mean cfp	Variance
BW *Escherichia coli* (ISO 9308-3)	Yes	N/A[2]	13563.0	20707.1	34270.1	491	442	59667.8
BW Intestinal Enterococci (ISO 7899-1)	Yes	N/A[2]	1790.5	80.3	1870.8	204	206	11346.5
DW *Clostridium perfringens* (ISO/WD 6461-2)	Yes	N/A[2]	34.8	107.2	142.0	65	56	47.5
DW Culturable organisms 22 °C (ISO 6222)	Yes	N/A[2]	28.7	7.8	36.5	58	57	19.7
DW Culturable organisms 36 °C (ISO 6222)	Yes	N/A[2]	23.8	13.6	37.4	56	55	30.4
DW *Escherichia coli* (ISO 9308-1)	Yes	N/A[2]	59.3	132.5	191.8	55	51	110.9
DW Intestinal enterococci (ISO 7899-2)	Yes	N/A[2]	47.7	15.1	62.8	53	52	18.7
DW *Pseudomonas aeruginosa* (prEN 12780)	No data available							

N/A[2] means that as the material is stable it is not appropriate to estimate the half life

Table 4.2 Short-term stability results for capsules

Temp °C	Stable	Estimated half life	Stability				Homogeneity	
			Short-term variance	Long-term variance	Total Variance	Mean cfp	Mean cfp	Variance
BW Escherichia Coli (ISO 9308-3)								
5	No	41 days	6403.4	24207.0	30610.4	386	442	59667.8
22	No	39 days	5338.4	15766.5	21104.9	236	442	59667.8
30	No	8 days	2907.7	20392.6	23300.4	183	442	59667.8
36	No	8 days	3109.1	22290.5	25399.6	181	442	59667.8
BW Intestinal Enterococci (ISO 7899-1)								
5	Yes	N/A[2]	3796.5	0.0[1]	3796.5	204	206	11346.5
22	Yes	N/A[2]	3514.5	265.4	3780.0	210	206	11346.5
30	Yes	N/A[2]	4696.2	0.0[1]	4696.2	205	206	11346.5
36	Yes	N/A[2]	6675.4	0.0[1]	6675.4	203	206	11346.5
DW Clostridium perfringens (ISO WD 6461-2)								
5	Yes	N/A[2]	28.6	115.1	143.7	58	56	47.5
22	Yes	N/A[2]	47.5	68.3	115.8	57	56	47.5
30	Yes	N/A[2]	39.1	42.0	81.1	59	56	47.5
36	Yes	N/A[2]	50.7	23.1	73.8	58	56	47.5
DW Culturable organisms 22°C (ISO 6222)								
5	Yes	N/A[2]	23.6	31.7	55.3	63	57	19.7
22	Yes	N/A[2]	37.6	6.3	43.9	63	57	19.7
30	Yes	N/A[2]	38.1	9.0	47.1	58	57	19.7
36	Yes	N/A[2]	43.3	6.2	49.6	56	57	19.7
DW Culturable organisms 36°C (ISO 6222)								
5	Yes	N/A[2]	27.4	9.0	36.4	56	55	30.4
22	Yes	N/A[2]	40.7	37.0	77.6	52	55	30.4
30	Yes	N/A[2]	26.5	18.9	45.4	53	55	30.4
36	Yes	N/A[2]	23.1	2.8	26.0	53	55	30.4
DW Escherichia coli (ISO 9308-1)								
5	No	60 days	23.5	209.8	233.3	37	51	110.9
22	No	20 days	18.5	193.0	211.5	25	51	110.9
30	No	10 days	13.6	181.9	195.5	21	51	110.9
36	No	6 days	13.3	241.9	255.2	21	51	110.9
DW Intestinal enterococci (ISO 7899-2)								
5	Yes	N/A[2]	39.3	15.0	54.3	55	52	18.7
22	Yes	N/A[2]	32.2	5.0	37.2	53	52	18.7
30	Yes	N/A[2]	43.5	5.1	48.7	50	52	18.7
36	Yes	N/A[2]	30.3	7.1	37.4	50	52	18.7
DW Pseudomonas aeruginosa (pr EN 12780)								
5	No data available							
22	No data available							
30	No data available							
36	No data available							

[1] Note the long-term variance values of 0 were estimated to be negative values. Obviously a negative variance is not possible so it has been replaced by a zero. The negative values occur due to the error in estimation.
N/A[2] means that as the material is stable it is not appropriate to estimate the half life

Annex 5 SOP BCR-water/001 (08-03-2002)

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 for 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 for 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 6 RIVM/MGB-I001 (26-02-2002)

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 analysed 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 analysed 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 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 and rinse with approximately 50 ml PS. 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 analysed 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 peptone saline solution (PS) of (5 ± 3) °C. 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 analysed 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 and rinse with approximately 50 ml PS. 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 analysed 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 and rinse with approximately 50 ml PS. 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 analysed 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 peptone saline solution (PS) of (5 ± 3) °C. 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

The procedure described in prEN 12780 will be analysed with gelatin capsules containing *Pseudomonas aeruginosa* (CIP 82118), batch LWL36-21/11/01, 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) – (4.00 ± 0.08) 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 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 and rinse with approximately 50 ml PS.

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 BCR-water/001 (05-10-2001). 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 7 Raw data homogeneity studies of the capsule RMs

Table 7.1 **Test batch *Escherichia coli* 6-2 -25/06/01**
Membrane filtration (0.5 ml of reconstituted capsule solution)on Laurylsulphate agar (LSA), ISO 9308-1:1990

Unit	count 1	count 2
1	62	59
2	71	56
3	55	52
4	55	59
5	41	50
6	57	60
7	65	53
8	53	54
9	55	71
10	48	60

Table 7.2 **Final batch *Escherichia coli* 6-2 -25/06/01**
Membrane filtration (0.5 ml of reconstituted capsule solution) on Laurylsulphate agar (LSA), ISO 9308-1:1990

Unit	count 1	count 2
1	52	48
2	37	25
3	28	24
4	27	39
5	41	35
6	31	37
7	26	33
8	37	19
9	39	32
10	40	42
11	21	34
12	35	26
13	42	33
14	26	33
15	36	21
16	40	46
17	42	42
18	36	33
19	47	47

Unit	count 1	count 2
20	37	48
21	25	35
22	48	33
23	34	28
24	27	32
25	39	35
26	36	35
27	48	29
28	49	55
29	23	44
30	38	36
31	35	33
32	38	38
33	32	26

Table 7.3 **Final batch *Escherichia coli* 6-2 -25/06/01**
Membrane filtration (0.5 ml of reconstituted capsule solution) on Lactose TTC agar with heptadecylsulphate (LTTC), ISO 9308-1: 2000

Unit	count 1	count 2	Unit	count 1	count 2
1	66	59	16	40	38
2	72	68	17	51	47
3	67	62	18	42	37
4	75	73	19	47	49
5	58	84	20	56	48
6	33	43	21	33	50
7	46	55	22	52	58
8	55	51	23	57	63
9	43	41	24	69	60
10	41	42	25	48	42
11	43	58	26	52	41
12	49	32	27	57	53
13	44	36	28	46	44
14	50	49	29	58	50
15	43	28	30	57	53

Table 7.4 **Final batch *Escherichia coli* 6-2 -25/06/01**
MPN (4 ml of reconstituted capsule solution) on microtitre plates, ISO 9308-3: 1998b

Unit	Replicate 1 MPN/ml	Replicate 2 MPN/ml	Unit	Replicate 1 MPN/ml	Replicate 2 MPN/ml
1	4.8	5.1	16	8.4	8.3
2	4.9	4.8	17	6.8	6.0
3	2.7	4.6	18	8.6	7.4
4	4.2	4.4	19	6.0	8.7
5	4.6	4.3	20	4.6	3.9
6	4.0	6.1	21	8.7	5.7
7	5.1	4.7	22	3.9	4.9
8	4.5	3.8	23	9.6	7.8
9	4.3	3.5	24	9.2	5.2
10	4.3	3.7	25	7.0	8.2
11	3.9	5.8	26	4.4	6.6
12	3.9	3.9	27	6.0	6.1
13	3.6	5.4	28	3.7	4.4
14	5.8	7.3	29	3.8	4.6
15	5.6	3.8	30	4.6	5.1

Table 7.5 **Test batch *Enterococcus faecium* LWL34-24/07/01**
Membrane filtration (1 ml of reconstituted capsule solution) on Kenner fecal agar (KFA), ISO 7899-2: 1984.

Unit	count 1	count 2	Unit	count 1	count 2
1	58	65	11	55	53
2	44	62	12	49	46
3	50	42	13	68	46
4	48	48	14	49	68
5	58	58	15	51	51
6	72	62	16	47	46
7	56	58	17	53	47
8	44	44	18	57	49
9	72	79	19	58	55
10	49	45	20	69	62

Table 7.6 **Final batch *Enterococcus faecium* LWL34-24/07/01**
Membrane filtration (1 ml of reconstituted capsule solution) on Slanetz and Bartley agar (S&B), ISO 7899-2: 2000.

Unit	count 1	count 2	Unit	count 1	count 2
1	38	45	16	47	54
2	54	49	17	52	51
3	58	49	18	59	63
4	47	54	19	60	58
5	55	48	20	48	54
6	48	56	21	56	49
7	47	50	22	56	56
8	38	57	23	64	47
9	48	61	24	51	59
10	48	62	25	49	57
11	44	59	26	49	58
12	49	57	27	55	44
13	41	62	28	50	46
14	47	57	29	42	38
15	58	46	30	54	62

Table 7.7 **Final batch *Enterococcus faecium* LWL34-24/07/01**
Pour plate (1 ml of reconstituted capsule solution) in Yeast Extract agar (YA), incubated at 22 °C, ISO 6222: 1999.

Unit	count 1	count 2	Unit	count 1	count 2
1	67	59	16	51	53
2	44	55	17	66	50
3	47	54	18	59	62
4	56	64	19	46	54
5	74	52	20	61	53
6	47	50	21	54	57
7	69	54	22	52	72
8	52	52	23	62	56
9	55	65	24	58	55
10	45	62	25	54	51
11	62	51	26	65	55
12	58	60	27	63	52
13	50	56	28	56	65
14	56	51	29	56	50
15	64	63	30	57	51

Table 7.8 **Final batch *Enterococcus faecium* LWL34-24/07/01**
Pour plate (1 ml of reconstituted capsule solution) in Yeast Extract agar (YA), incubated at 36 °C, ISO 6222: 1999.

Unit	count 1	count 2	Unit	count 1	count 2
1	56	60	16	51	45
2	48	54	17	53	59
3	53	75	18	45	52
4	51	55	19	49	60
5	59	68	20	56	60
6	42	34	21	58	54
7	55	52	22	52	63
8	63	52	23	65	52
9	51	48	24	50	67
10	54	50	25	54	58
11	65	48	26	54	62
12	46	54	27	55	73
13	58	51	28	45	57
14	46	64	29	58	68
15	52	69	30	66	48

Table 7.9 **Final batch *Enterococcus faecium* LWL34-24/07/01**
MPN (3 ml of reconstituted capsule solution) on microtitre plates, ISO 7899-1: 1998a

Unit	Replicate 1 MPN/ml	Replicate 2 MPN/ml
1	2.0	2.1
2	2.0	1.4
3	2.5	3.1
4	1.4	2.0
5	1.1	0.9
6	1.6	2.3
7	1.8	2.2
8	1.1	2.3
9	0.9	1.3
10	1.6	2.2
11	1.4	2.2
12	3.3	3.7
13	2.2	3.3
14	1.4	3.3
15	2.0	2.5

Unit	Replicate 1 MPN/ml	Replicate 2 MPN/ml
16	1.8	1.8
17	1.8	1.8
18	2.9	3.2
19	0.9	2.2
20	2.1	1.8
21	1.3	2.7
22	2.5	2.2
23	1.1	2.7
24	2.2	1.8
25	1.9	2.3
26	1.4	1.8
27	2.7	2.1
28	1.9	1.8
29	1.8	1.8
30	2.3	2.0

Table 7.10 **Test batch *Clostridium perfringens* LWL3501-24/10/01**
Membrane filtration (1 ml of reconstituted capsule solution) on Tryptose Cycloserine agar (TSC), ISO/WD 6461-2: 2001.

Unit	count 1	count 2
1	41	67
2	68	61
3	59	54
4	62	57
5	55	58
6	58	71
7	63	53
8	60	50
9	54	38
10	45	54

Table 7.11 **Final batch *Clostridium perfringens* LWL3501-24/10/01**
Membrane filtration (1 ml of reconstituted capsule solution) on Tryptose Cycloserine agar (TSC), ISO/WD 6461-2: 2001.

Unit	count 1	count 2
1	31	56
2	55	48
3	45	57
4	40	50
5	42	70
6	55	56
7	53	64
8	49	76
9	45	66
10	59	57
11	51	58
12	69	55
13	54	57
14	47	53
15	61	53
16	48	42
17	50	42
18	57	70
19	52	73
20	57	71
21	65	49
22	34	68
23	57	83
24	48	39
25	61	68
26	59	60
27	52	58
28	62	64
29	53	64
30	66	56

Table 7.12 **Test batch *Pseudomonas fluorescenss* LWL36-21/11/01**
Membrane filtration (1 ml of reconstituted capsule solution) on CNagar, prEN 12780: 1999.

Unit	count 1	count 2
1	11	19
2	14	10
3	21	17
4	12	18
5	16	15
6	11	23
7	11	13
8	18	12
9	16	10
10	10	18

Table 7.13 **Final batch *Pseudomonas fluorescenss* LWL36-21/11/01**
Membrane filtration (2 ml of reconstituted capsule solution) on CNagar, prEN 12780: 1999.

Unit	count 1	count 2
1	12	7
2	7	16
3	10	7
4	7	14
5	8	11
6	12	8
7	13	7
8	10	6
9	3	10
10	6	8

Annex 8 Raw data long-term stability studies of the capsule RMs

Table 8.1 **Long-term stability study of batch *Escherichia coli* 6-2 -25/06/01, stored at –20 °C. Membrane filtration (0.5 ml of reconstituted capsule solution) on Lactose TTC agar with heptadecylsulphate, ISO 9308-1: 2000**

Date	Unit	Dupl.	Count	Date	Unit	Dupl.	Count	Date	Unit	Dupl.	Count
31-10-2001	1	1	66	11-04-2002	1	1	41	09-09-2002	1	1	38
	1	2	59		1	2	36		1	2	39
	2	1	72		2	1	51		2	1	32
	2	2	68		2	2	52		2	2	58
	3	1	67		3	1	56		3	1	37
	3	2	62		3	2	45		3	2	32
	4	1	75		4	1	55		4	1	53
	4	2	73		4	2	38		4	2	58
	5	1	58		5	1	44		5	1	35
	5	2	84		5	2	47		5	2	32
26-11-2001	1	1	50	18-06-2002	1	1	61	07-10-2002	1	1	71
	1	2	57		1	2	62		1	2	50
	2	1	76		2	1	52		2	1	47
	2	2	84		2	2	65		2	2	38
	3	1	74		3	1	61		3	1	53
	3	2	74		3	2	65		3	2	59
	4	1	77		4	1	59		4	1	68
	4	2	62		4	2	57		4	2	57
	5	1	75		5	1	65		5	1	51
	5	2	61		5	2	50		5	2	72
07-01-2002	1	1	34	04-07-2002	1	1	48	04-11-2002	1	1	33
	1	2	46		1	2	55		1	2	38
	2	1	59		2	1	35		2	1	58
	2	2	40		2	2	48		2	2	52
	3	1	38		3	1	43		3	1	35
	3	2	51		3	2	40		3	2	31
	4	1	58		4	1	33		4	1	52
	4	2	36		4	2	42		4	2	42
	5	1	55		5	1	42		5	1	34
	5	2	47		5	2	33		5	2	42
07-03-2002	1	1	55	08-08-2002	1	1	53	02-12-2002	1	1	87
	1	2	56		1	2	56		1	2	82
	2	1	84		2	1	49		2	1	69
	2	2	68		2	2	51		2	2	61
	3	1	69		3	1	58		3	1	69
	3	2	64		3	2	66		3	2	76
	4	1	56		4	1	55		4	1	80
	4	2	58		4	2	43		4	2	59
	5	1	65		5	1	34		5	1	76
	5	2	74		5	2	27		5	2	85

Table 8.2 **Long-term stability study of batch *Escherichia coli* 6-2 -25/06/01, stored at –20 °C. MPN (4 ml of reconstituted capsule solution) on microtitre plates, ISO 9308-3: 1998b. MPN/ 100 ml are given.**

Date	Unit	Dupl.	Count
19-11-2001	1	1	485
	1	2	509
	2	1	489
	2	2	485
	3	1	272
	3	2	461
	4	1	415
	4	2	438
	5	1	461
	5	2	430
18-12-2001	1	1	397
	1	2	368
	2	1	461
	2	2	485
	3	1	419
	3	2	449
	4	1	408
	4	2	419
	5	1	327
	5	2	434
06-03-2002	1	1	289
	1	2	143
	2	1	197
	2	2	234
	3	1	197
	3	2	195
	4	1	94
	4	2	144
	5	1	179
	5	2	197
16-04-2002	1	1	270
	1	2	327
	2	1	612
	2	2	353
	3	1	591
	3	2	675
	4	1	353
	4	2	393
	5	1	559
	5	2	534
18-06-2002	1	1	612
	1	2	419
	2	1	612
	2	2	419
	3	1	580
	3	2	457
	4	1	461
	4	2	514
	5	1	981
	5	2	814
04-07-2002	1	1	415
	1	2	627
	2	1	371
	2	2	371
	3	1	347
	3	2	375
	4	1	312
	4	2	393
	5	1	438
	5	2	312
08-08-2002	1	1	509
	1	2	668
	2	1	872
	2	2	534
	3	1	1092
	3	2	668
	4	1	647
	4	2	375
	5	1	393
	5	2	393
09-09-2002	1	1	485
	1	2	438
	2	1	327
	2	2	350
	3	1	534
	3	2	371
	4	1	465
	4	2	415
	5	1	465
	5	2	612
07-10-2002	1	1	559
	1	2	485
	2	1	419
	2	2	509
	3	1	654
	3	2	640
	4	1	796
	4	2	782
	5	1	1254
	5	2	419
13-11-2002	1	1	397
	1	2	287
	2	1	438
	2	2	509
	3	1	509
	3	2	393
	4	1	534
	4	2	472
	5	1	393
	5	2	393
02-12-2002	1	1	875
	1	2	968
	2	1	574
	2	2	787
	3	1	705
	3	2	704
	4	1	580
	4	2	585
	5	1	675
	5	2	1076

Table 8.3 **Long-term stability study of batch *Enterococcus faecium* LWL34-24/07/01 stored at –20 °C. Membrane filtration (1 ml of reconstituted capsule solution) on Slanetz and Bartley agar, ISO 7899-2: 2000**

Date	Unit	Dupl.	Count
24-09-2001	1	1	38
	1	2	45
	2	1	54
	2	2	49
	3	1	58
	3	2	49
	4	1	47
	4	2	54
	5	1	55
	5	2	48
28-11-2001	1	1	39
	1	2	55
	2	1	58
	2	2	50
	3	1	41
	3	2	47
	4	1	59
	4	2	59
	5	1	50
	5	2	45
18-12-2001	1	1	45
	1	2	50
	2	1	47
	2	2	55
	3	1	55
	3	2	49
	4	1	53
	4	2	63
	5	1	37
	5	2	47
06-02-2002	1	1	42
	1	2	51
	2	1	62
	2	2	53
	3	1	57
	3	2	69
	4	1	61
	4	2	50
	5	1	72
	5	2	51
06-03-2002	1	1	64
	1	2	48
	2	1	48
	2	2	66
	3	1	62
	3	2	57
	4	1	48
	4	2	58
	5	1	48
	5	2	57
10-04-2002	1	1	56
	1	2	62
	2	1	64
	2	2	59
	3	1	62
	3	2	55
	4	1	56
	4	2	65
	5	1	81
	5	2	76
10-06-2002	1	1	61
	1	2	54
	2	1	58
	2	2	53
	3	1	57
	3	2	61
	4	1	54
	4	2	60
	5	1	58
	5	2	56
05-07-2002	1	1	64
	1	2	63
	2	1	45
	2	2	37
	3	1	64
	3	2	53
	4	1	45
	4	2	47
	5	1	46
	5	2	49
08-08-2002	1	1	61
	1	2	57
	2	1	49
	2	2	46
	3	1	40
	3	2	25
	4	1	35
	4	2	38
	5	1	48
	5	2	37
09-09-2002	1	1	44
	1	2	47
	2	1	56
	2	2	50
	3	1	40
	3	2	63
	4	1	64
	4	2	58
	5	1	53
	5	2	66
07-10-2002	1	1	37
	1	2	54
	2	1	52
	2	2	62
	3	1	51
	3	2	45
	4	1	48
	4	2	56
	5	1	53
	5	2	50
04-11-2002	1	1	70
	1	2	63
	2	1	53
	2	2	40
	3	1	47
	3	2	53
	4	1	55
	4	2	50
	5	1	59
	5	2	54
02-12-2002	1	1	70
	1	2	71
	2	1	65
	2	2	52
	3	1	55
	3	2	70
	4	1	54
	4	2	44
	5	1	55
	5	2	40

Table 8.4 **Long-term stability study of batch *Enterococcus faecium* LWL34-24/07/01 stored at –20 °C. Pour plate (1 ml of reconstituted capsule solution) in Yeast Extract agar agar, incubated at 22 °C, ISO 6222: 1999**

Date	Unit	Dupl.	Count
15-10-2001	1	1	67
	1	2	59
	2	1	44
	2	2	55
	3	1	47
	3	2	54
	4	1	56
	4	2	64
	5	1	74
	5	2	52
28-11-2001	1	1	55
	1	2	59
	2	1	55
	2	2	66
	3	1	52
	3	2	52
	4	1	58
	4	2	64
	5	1	52
	5	2	65
18-12-2001	1	1	61
	1	2	58
	2	1	54
	2	2	65
	3	1	56
	3	2	63
	4	1	58
	4	2	52
	5	1	54
	5	2	46
06-02-2002	1	1	47
	1	2	60
	2	1	53
	2	2	49
	3	1	65
	3	2	55
	4	1	48
	4	2	42
	5	1	62
	5	2	61
06-03-2002	1	1	56
	1	2	62
	2	1	41
	2	2	54
	3	1	51
	3	2	49
	4	1	52
	4	2	53
	5	1	56
	5	2	55
17-04-2002	1	1	55
	1	2	58
	2	1	58
	2	2	41
	3	1	56
	3	2	54
	4	1	60
	4	2	58
	5	1	58
	5	2	51
10-06-2002	1	1	63
	1	2	63
	2	1	58
	2	2	57
	3	1	59
	3	2	71
	4	1	60
	4	2	61
	5	1	57
	5	2	61
05-07-2002	1	1	72
	1	2	70
	2	1	54
	2	2	52
	3	1	80
	3	2	56
	4	1	60
	4	2	77
	5	1	61
	5	2	65
08-08-2002	1	1	56
	1	2	62
	2	1	57
	2	2	66
	3	1	51
	3	2	57
	4	1	54
	4	2	44
	5	1	68
	5	2	65
09-09-2002	1	1	51
	1	2	65
	2	1	68
	2	2	68
	3	1	50
	3	2	50
	4	1	57
	4	2	58
	5	1	64
	5	2	53
07-10-2002	1	1	64
	1	2	52
	2	1	55
	2	2	59
	3	1	56
	3	2	58
	4	1	56
	4	2	47
	5	1	51
	5	2	59
04-11-2002	1	1	54
	1	2	51
	2	1	68
	2	2	60
	3	1	49
	3	2	50
	4	1	54
	4	2	67
	5	1	58
	5	2	51
02-12-2002	1	1	73
	1	2	63
	2	1	59
	2	2	74
	3	1	63
	3	2	67
	4	1	78
	4	2	61
	5	1	52
	5	2	59

Table 8.5 **Long-term stability study of batch *Enterococcus faecium* LWL34-24/07/01 stored at –20 °C. Pour plate (1 ml of reconstituted capsule solution) in Yeast Extract agar agar, incubated at 36 °C, ISO 6222: 1999**

Date	Unit	Dupl.	Count
15-10-2001	1	1	56
	1	2	60
	2	1	48
	2	2	54
	3	1	53
	3	2	75
	4	1	51
	4	2	55
	5	1	59
	5	2	68
28-11-2001	1	1	48
	1	2	62
	2	1	66
	2	2	69
	3	1	56
	3	2	52
	4	1	55
	4	2	46
	5	1	55
	5	2	57
18-12-2001	1	1	48
	1	2	55
	2	1	55
	2	2	64
	3	1	51
	3	2	48
	4	1	46
	4	2	51
	5	1	52
	5	2	52
06-02-2002	1	1	52
	1	2	58
	2	1	50
	2	2	50
	3	1	47
	3	2	56
	4	1	50
	4	2	42
	5	1	54
	5	2	66
06-03-2002	1	1	54
	1	2	62
	2	1	40
	2	2	63
	3	1	60
	3	2	48
	4	1	50
	4	2	63
	5	1	57
	5	2	47
17-04-2002	1	1	39
	1	2	36
	2	1	50
	2	2	43
	3	1	54
	3	2	43
	4	1	45
	4	2	40
	5	1	47
	5	2	54
18-06-2002	1	1	57
	1	2	51
	2	1	61
	2	2	64
	3	1	51
	3	2	63
	4	1	69
	4	2	53
	5	1	65
	5	2	66
05-07-2002	1	1	61
	1	2	46
	2	1	54
	2	2	45
	3	1	68
	3	2	57
	4	1	61
	4	2	70
	5	1	59
	5	2	58
08-08-2002	1	1	69
	1	2	53
	2	1	44
	2	2	57
	3	1	54
	3	2	60
	4	1	46
	4	2	54
	5	1	61
	5	2	48
09-09-2002	1	1	59
	1	2	53
	2	1	63
	2	2	55
	3	1	51
	3	2	56
	4	1	55
	4	2	64
	5	1	50
	5	2	54
07-10-2002	1	1	52
	1	2	50
	2	1	72
	2	2	51
	3	1	62
	3	2	54
	4	1	53
	4	2	50
	5	1	65
	5	2	57
04-11-2002	1	1	60
	1	2	54
	2	1	63
	2	2	64
	3	1	61
	3	2	59
	4	1	51
	4	2	57
	5	1	52
	5	2	69
02-12-2002	1	1	66
	1	2	63
	2	1	67
	2	2	59
	3	1	68
	3	2	66
	4	1	52
	4	2	60
	5	1	58
	5	2	63

Table 8.6 **Long-term stability study of batch *Enterococcus faecium* LWL34-24/07/01 stored at –20 °C. MPN (3 ml of reconstituted capsule solution) on microtitre plates, ISO 7899-1: 1998a. MPN/ 100 ml are given.**

Date	Unit	Dupl.	Count
20-11-2001	1	1	197
	1	2	213
	2	1	195
	2	2	142
	3	1	253
	3	2	309
	4	1	143
	4	2	197
	5	1	110
	5	2	94
18-12-2001	1	1	233
	1	2	179
	2	1	177
	2	2	251
	3	1	179
	3	2	161
	4	1	160
	4	2	195
	5	1	215
	5	2	161
06-02-2002	1	1	253
	1	2	143
	2	1	251
	2	2	144
	3	1	126
	3	2	272
	4	1	215
	4	2	144
	5	1	251
	5	2	161
06-03-2002	1	1	289
	1	2	143
	2	1	197
	2	2	234
	3	1	197
	3	2	195
	4	1	99
	4	2	144
	5	1	179
	5	2	197
16-04-2002	1	1	232
	1	2	230
	2	1	251
	2	2	215
	3	1	266
	3	2	195
	4	1	144
	4	2	179
	5	1	109
	5	2	177
22-05-2002	1	1	292
	1	2	195
	2	1	177
	2	2	110
	3	1	234
	3	2	234
	4	1	251
	4	2	289
	5	1	309
	5	2	197
18-06-2002	1	1	234
	1	2	234
	2	1	309
	2	2	144
	3	1	177
	3	2	289
	4	1	215
	4	2	127
	5	1	197
	5	2	197
05-07-2002	1	1	368
	1	2	287
	2	1	212
	2	2	215
	3	1	368
	3	2	127
	4	1	144
	4	2	287
	5	1	232
	5	2	194
08-08-2002	1	1	213
	1	2	353
	2	1	144
	2	2	213
	3	1	213
	3	2	160
	4	1	177
	4	2	179
	5	1	213
	5	2	179
09-09-2002	1	1	179
	1	2	197
	2	1	270
	2	2	292
	3	1	268
	3	2	272
	4	1	161
	4	2	272
	5	1	127
	5	2	309
07-10-2002	1	1	179
	1	2	127
	2	1	232
	2	2	179
	3	1	110
	3	2	144
	4	1	234
	4	2	160
	5	1	179
	5	2	251
13-11-2002	1	1	213
	1	2	251
	2	1	307
	2	2	251
	3	1	161
	3	2	144
	4	1	160
	4	2	232
	5	1	213
	5	2	195
02-12-2002	1	1	215
	1	2	194
	2	1	143
	2	2	247
	3	1	270
	3	2	215
	4	1	109
	4	2	160
	5	1	61
	5	2	195

Table 8.7 **Long-term stability study of batch *Clostridium perfringens* LWL3501-24/10/01 stored at –20 °C. Membrane filtration (1 ml of reconstituted capsule solution) on Tryptose Cycloserine agar, ISO 6461-2: 2001**

Date	Unit	Dupl.	Count
07-11-2001	1	1	31
	1	2	56
	2	1	55
	2	2	48
	3	1	45
	3	2	57
	4	1	40
	4	2	50
	5	1	42
	5	2	70
03-12-2001	1	1	76
	1	2	64
	2	1	63
	2	2	71
	3	1	64
	3	2	62
	4	1	61
	4	2	53
	5	1	72
	5	2	66
10-01-2002	1	1	57
	1	2	66
	2	1	61
	2	2	51
	3	1	58
	3	2	59
	4	1	71
	4	2	70
	5	1	56
	5	2	69
06-03-2002	1	1	56
	1	2	54
	2	1	57
	2	2	39
	3	1	32
	3	2	39
	4	1	39
	4	2	39
	5	1	60
	5	2	53
16-04-2002	1	1	78
	1	2	88
	2	1	83
	2	2	83
	3	1	84
	3	2	79
	4	1	91
	4	2	68
	5	1	73
	5	2	79
18-06-2002	1	1	60
	1	2	62
	2	1	54
	2	2	51
	3	1	47
	3	2	58
	4	1	64
	4	2	59
	5	1	64
	5	2	56
04-07-2002	1	1	84
	1	2	72
	2	1	79
	2	2	78
	3	1	69
	3	2	81
	4	1	87
	4	2	62
	5	1	66
	5	2	74
08-08-2002	1	1	58
	1	2	78
	2	1	65
	2	2	63
	3	1	78
	3	2	78
	4	1	80
	4	2	83
	5	1	68
	5	2	83
09-09-2002	1	1	71
	1	2	75
	2	1	73
	2	2	68
	3	1	74
	3	2	81
	4	1	84
	4	2	72
	5	1	78
	5	2	70
07-10-2002	1	1	83
	1	2	74
	2	1	67
	2	2	76
	3	1	63
	3	2	60
	4	1	72
	4	2	62
	5	1	71
	5	2	69
04-11-2002	1	1	68
	1	2	68
	2	1	67
	2	2	65
	3	1	74
	3	2	71
	4	1	71
	4	2	87
	5	1	84
	5	2	55
02-12-2002	1	1	48
	1	2	58
	2	1	49
	2	2	55
	3	1	49
	3	2	53
	4	1	62
	4	2	80
	5	1	57
	5	2	70

Table 8.8 **Long-term stability study of batch *Pseudomonas aeruginosa* LWL36-21/11/01 stored at –20 °C. Membrane filtration (1-4 ml of reconstituted capsule solution) on CN-agar, prEN 12780: 1999**

Date	Unit	Dupl.	Count
21-11-2001	1	1	11
	1	2	19
	2	1	14
	2	2	10
	3	1	21
	3	2	17
	4	1	12
	4	2	18
1 ml	5	1	16
caps.	5	2	15
sol.	6	1	11
	6	2	23
	7	1	11
	7	2	13
	8	1	18
	8	2	12
	9	1	16
	9	2	10
	10	1	10
	10	2	18
05-12-2001	1	1	20
	1	2	24
	2	1	26
	2	2	21
	3	1	16
	3	2	22
	4	1	17
	4	2	19
	5	1	23
2 ml	5	2	26
caps.	6	1	17
sol.	6	2	34
	7	1	21
	7	2	18
	8	1	29
	8	2	20
	9	1	21
	9	2	22
	10	1	19
	10	2	23
12-12-2001	1	1	12
	1	2	7
	2	1	7
	2	2	16
	3	1	10
2 ml	3	2	7
caps.	4	1	7
sol.	4	2	14
	5	1	8
	5	2	11

Date	Unit	Dupl.	Count
12-12-2001 (cont.)	6	1	12
	6	2	8
	7	1	13
	7	2	7
	8	1	10
2 ml	8	2	6
caps.	9	1	3
sol.	9	2	10
	10	1	6
	10	2	8
09-01-2002	1	1	5
	1	2	11
	2	1	10
	2	2	12
	3	1	12
	3	2	17
	4	1	6
	4	2	13
3 ml	5	1	11
caps.	5	2	8
sol.	6	1	9
	6	2	10
	7	1	6
	7	2	12
	8	1	14
	8	2	16
	9	1	5
	9	2	8
	10	1	11
	10	2	15
14-01-2001	1	1	11
	1	2	8
	2	1	16
	2	2	13
	3	1	12
	3	2	7
	4	1	7
	4	2	7
	5	1	12
4 ml	5	2	9
caps.	6	1	11
sol.	6	2	11
	7	1	9
	7	2	8
	8	1	6
	8	2	8
	9	1	11
	9	2	11
	10	1	13
	10	2	14

Date	Unit	Dupl.	Count
08-04-2002	1	1	4
	1	2	12
	2	1	4
	2	2	7
	3	1	7
	3	2	6
	4	1	7
	4	2	5
	5	1	6
4 ml	5	2	6
caps.	6	1	10
sol.	6	2	6
	7	1	4
	7	2	7
	8	1	5
	8	2	8
	9	1	4
	9	2	2
	10	1	3
	10	2	7
17-06-2002	1	1	5
	1	2	5
	2	1	6
	2	2	15
4 ml	3	1	15
caps.	3	2	12
sol.	4	1	8
	4	2	10
	5	1	5
	5	2	7

Annex 9 Raw data short-term stability studies of the capsule RMs

Table 9.1 **Short-term stability study of batch *Escherichia coli* 6-2 -25/06/01, stored at 5 °C. Membrane filtration (0.5 ml of reconstituted capsule solution) on Lactose TTC agar with heptadecylsulphate, ISO 9308-1: 2000**

Day	Date	Unit	Duplicate	Count	Day	Date	Unit	Duplicate	Count
0	18-3-02	1	1	53	57	14-5-02	1	1	24
		1	2	39			1	2	25
		2	1	47			2	1	19
		2	2	48			2	2	26
		3	1	54			3	1	28
		3	2	52			3	2	49
		4	1	44			4	1	33
		4	2	47			4	2	39
		5	1	50			5	1	36
		5	2	38			5	2	38
1	19-3-02	1	1	75	77	3-6-02	1	1	24
		1	2	72			1	2	28
		2	1	64			2	1	22
		2	2	54			2	2	32
		3	1	72			3	1	26
		3	2	61			3	2	26
		4	1	69			4	1	32
		4	2	73			4	2	22
		5	1	78			5	1	22
		5	2	62			5	2	12
16	3-4-02	1	1	49	84	10-6-02	1	1	21
		1	2	62			1	2	20
		2	1	50			2	1	19
		2	2	47			2	2	22
		3	1	52			3	1	30
		3	2	48			3	2	22
		4	1	60			4	1	11
		4	2	51			4	2	18
		5	1	37			5	1	16
		5	2	45			5	2	20
28	15-4-02	1	1	32	100	26-6-02	1	1	25
		1	2	37			1	2	29
		2	1	49			2	1	26
		2	2	33			2	2	21
		3	1	45			3	1	27
		3	2	44			3	2	18
		4	1	29			4	1	25
		4	2	46			4	2	26
		5	1	34			5	1	14
		5	2	31			5	2	26
49	6-5-02	1	1	39	112	8-7-02	1	1	33
		1	2	49			1	2	21
		2	1	36			2	1	28
		2	2	37			2	2	33
		3	1	33			3	1	29
		3	2	35			3	2	36
		4	1	44			4	1	35
		4	2	43			4	2	35
		5	1	42			5	1	37
		5	2	46			5	2	26

Table 9.2 **Short-term stability study of batch *Escherichia coli* 6-2 -25/06/01, stored at 22 °C. Membrane filtration (0.5 ml of reconstituted capsule solution) on Lactose TTC agar with heptadecylsulphate, ISO 9308-1: 2000**

Day	Date	Unit	Duplicate	Count	Day	Date	Unit	Duplicate	Count
0	18-3-02	1	1	53	28	15-4-02	1	1	8
		1	2	39			1	2	17
		2	1	47			2	1	14
		2	2	48			2	2	17
		3	1	54			3	1	15
		3	2	52			3	2	23
		4	1	44			4	1	17
		4	2	47			4	2	21
		5	1	50			5	1	21
		5	2	38			5	2	12
1	19-3-02	1	1	61	35	22-4-02	1	1	14
		1	2	52			1	2	16
		2	1	54			2	1	9
		2	2	54			2	2	8
		3	1	34			3	1	7
		3	2	36			3	2	13
		4	1	44			4	1	11
		4	2	54			4	2	15
		5	1	49			5	1	7
		5	2	41			5	2	18
7	25-3-02	1	1	28	49	6-5-02	1	1	13
		1	2	33			1	2	17
		2	1	27			2	1	9
		2	2	27			2	2	12
		3	1	24			3	1	8
		3	2	21			3	2	9
		4	1	27			4	1	9
		4	2	23			4	2	4
		5	1	30			5	1	17
		5	2	22			5	2	8
16	3-4-02	1	1	18	57	14-5-02	1	1	12
		1	2	27			1	2	8
		2	1	21			2	1	13
		2	2	30			2	2	8
		3	1	22			3	1	21
		3	2	30			3	2	13
		4	1	29			4	1	11
		4	2	32			4	2	11
		5	1	29			5	1	16
		5	2	32			5	2	17
21	8-4-02	1	1	28	64	21-5-02	1	1	16
		1	2	36			1	2	17
		2	1	28			2	1	18
		2	2	37			2	2	12
		3	1	22			3	1	10
		3	2	20			3	2	11
		4	1	31			4	1	14
		4	2	33			4	2	13
		5	1	36			5	1	17
		5	2	40			5	2	15

Table 9.3 **Short-term stability study of batch *Escherichia coli* 6-2 -25/06/01, stored at 30 °C. Membrane filtration (0.5 ml of reconstituted capsule solution) on Lactose TTC agar with heptadecylsulphate, ISO 9308-1: 2000**

Day	Date	Unit	Duplicate	Count
0	18-3-02	1	1	53
		1	2	39
		2	1	47
		2	2	48
		3	1	54
		3	2	52
		4	1	44
		4	2	47
		5	1	50
		5	2	38
1	19-3-02	1	1	49
		1	2	49
		2	1	52
		2	2	56
		3	1	45
		3	2	42
		4	1	40
		4	2	41
		5	1	39
		5	2	32
4	22-3-02	1	1	35
		1	2	35
		2	1	33
		2	2	33
		3	1	25
		3	2	21
		4	1	31
		4	2	36
		5	1	26
		5	2	28
7	25-3-02	1	1	22
		1	2	23
		2	1	15
		2	2	19
		3	1	21
		3	2	16
		4	1	17
		4	2	19
		5	1	25
		5	2	21
10	28-3-02	1	1	17
		1	2	10
		2	1	12
		2	2	10
		3	1	15
		3	2	18
		4	1	12
		4	2	16
		5	1	17
		5	2	21
16	3-4-02	1	1	23
		1	2	22
		2	1	15
		2	2	17
		3	1	19
		3	2	15
		4	1	22
		4	2	20
		5	1	21
		5	2	15
18	5-4-02	1	1	13
		1	2	14
		2	1	10
		2	2	10
		3	1	16
		3	2	17
		4	1	12
		4	2	14
		5	1	14
		5	2	17
21	8-4-02	1	1	7
		1	2	7
		2	1	8
		2	2	12
		3	1	13
		3	2	12
		4	1	10
		4	2	13
		5	1	14
		5	2	15
24	11-4-02	1	1	4
		1	2	12
		2	1	8
		2	2	9
		3	1	10
		3	2	4
		4	1	17
		4	2	16
		5	1	11
		5	2	6
28	15-4-02	1	1	13
		1	2	11
		2	1	8
		2	2	9
		3	1	8
		3	2	10
		4	1	8
		4	2	8
		5	1	10
		5	2	6
31	18-4-02	1	1	5
		1	2	16
		2	1	11
		2	2	18
		3	1	15
		3	2	12
		4	1	14
		4	2	15
		5	1	17
		5	2	12

Table 9.4 **Short-term stability study of batch *Escherichia coli* 6-2 -25/06/01, stored at 36 °C. Membrane filtration (0.5 ml of reconstituted capsule solution) on Lactose TTC agar with heptadecylsulphate, ISO 9308-1: 2000**

Day	Date	Unit	Duplicate	Count
0	18-3-02	1	1	53
		1	2	39
		2	1	47
		2	2	48
		3	1	54
		3	2	52
		4	1	44
		4	2	47
		5	1	50
		5	2	38
1	19-3-02	1	1	29
		1	2	40
		2	1	39
		2	2	40
		3	1	42
		3	2	49
		4	1	47
		4	2	39
		5	1	45
		5	2	40
2	20-3-02	1	1	28
		1	2	27
		2	1	43
		2	2	24
		3	1	48
		3	2	28
		4	1	33
		4	2	33
		5	1	39
		5	2	37
3	21-3-02	1	1	33
		1	2	35
		2	1	45
		2	2	47
		3	1	41
		3	2	31
		4	1	21
		4	2	38
		5	1	35
		5	2	37
4	22-3-02	1	1	15
		1	2	26
		2	1	19
		2	2	15
		3	1	25
		3	2	33
		4	1	29
		4	2	28
		5	1	18
		5	2	14
7	25-3-02	1	1	12
		1	2	14
		2	1	11
		2	2	8
		3	1	11
		3	2	5
		4	1	9
		4	2	7
		5	1	14
		5	2	14
8	26-3-02	1	1	11
		1	2	10
		2	1	15
		2	2	14
		3	1	6
		3	2	18
		4	1	15
		4	2	15
		5	1	22
		5	2	8
9	27-3-02	1	1	12
		1	2	8
		2	1	9
		2	2	10
		3	1	9
		3	2	8
		4	1	12
		4	2	4
		5	1	8
		5	2	16
10	28-3-02	1	1	6
		1	2	9
		2	1	11
		2	2	7
		3	1	2
		3	2	9
		4	1	11
		4	2	13
		5	1	10
		5	2	4
11	29-3-02	1	1	4
		1	2	4
		2	1	6
		2	2	7
		3	1	6
		3	2	6
		4	1	3
		4	2	9
		5	1	10
		5	2	3
16	3-4-02	1	1	6
		1	2	5
		2	1	0
		2	2	7
		3	1	5
		3	2	5
		4	1	8
		4	2	4
		5	1	3
		5	2	5

Table 9.5 **Short-term stability study of batch *Escherichia coli* 6-2 -25/06/01, stored at 5 °C. MPN (4 ml of reconstituted capsule solution) on microtitre plates, ISO 9308-3: 1998b. MPN/ 100 ml are given (only singular counts were made).**

Day	Date	Unit	Count
0	18-3-02	1	633
		2	350
		3	485
		4	509
		5	509
1	19-3-02	1	675
		2	640
		3	529
		4	791
		5	612
16	3-4-02	1	580
		2	661
		3	690
		4	585
		5	612
28	15-4-02	1	268
		2	304
		3	415
		4	480
		5	461
49	6-5-02	1	438
		2	539
		3	353
		4	485
		5	272
57	14-5-02	1	144
		2	272
		3	332
		4	144
		5	197
77	3-6-02	1	197
		2	270
		3	234
		4	179
		5	438
84	10-6-02	1	270
		2	213
		3	249
		4	230
		5	234
100	26-6-02	1	195
		2	195
		3	292
		4	270
		5	272
112	8-7-02	1	253
		2	312
		3	312
		4	371
		5	347

Table 9.6 **Short-term stability study of batch *Escherichia coli* 6-2 -25/06/01, stored at 22 °C. MPN (4 ml of reconstituted capsule solution) on microtitre plates, ISO 9308-3: 1998b. MPN/ 100 ml are given (only singular counts were made).**

Day	Date	Unit	Count
0	18-3-02	1	633
		2	350
		3	485
		4	509
		5	509
1	19-3-02	1	292
		2	500
		3	393
		4	312
		5	371
7	25-3-02	1	161
		2	332
		3	215
		4	212
		5	332
16	3-4-02	1	234
		2	234
		3	215
		4	197
		5	197
21	8-4-02	1	215
		2	287
		3	232
		4	371
		5	480
28	15-4-02	1	215
		2	195
		3	232
		4	270
		5	234
35	22-4-02	1	177
		2	234
		3	195
		4	46
		5	93
49	6-5-02	1	110
		2	94
		3	94
		4	46
		5	46
57	14-5-02	1	15
		2	177
		3	289
		4	161
		5	94
64	21-5-02	1	94
		2	110
		3	94
		4	127
		5	109

Table 9.7 **Short-term stability study of batch *Escherichia coli* 6-2 -25/06/01, stored at 30 °C. MPN (4 ml of reconstituted capsule solution) on microtitre plates, ISO 9308-3: 1998b. MPN/ 100 ml are given (only singular counts were made).**

Day	Date	Unit	Count
0	18-3-02	1	633
		2	350
		3	485
		4	509
		5	509
1	19-3-02	1	442
		2	312
		3	397
		4	442
		5	350
4	22-3-02	1	197
		2	215
		3	234
		4	215
		5	393
7	25-3-02	1	179
		2	161
		3	161
		4	215
		5	253
10	28-3-02	1	143
		2	61
		3	127
		4	213
		5	77
16	3-4-02	1	197
		2	197
		3	144
		4	144
		5	144
18	5-4-02	1	93
		2	144
		3	127
		4	197
		5	144
21	8-4-02	1	30
		2	94
		3	144
		4	45
		5	127
24	11-4-02	1	77
		2	110
		3	61
		4	15
		5	110
28	15-4-02	1	30
		2	30
		3	61
		4	77
		5	46
31	18-4-02	1	30
		2	61
		3	46
		4	30
		5	46

Table 9.8 **Short-term stability study of batch *Escherichia coli* 6-2 -25/06/01, stored at 36 °C. MPN (4 ml of reconstituted capsule solution) on microtitre plates, ISO 9308-3: 1998b. MPN/ 100 ml are given (only singular counts were made).**

Day	Date	Unit	Count
0	18-3-02	1	633
		2	350
		3	485
		4	509
		5	509
1	19-3-02	1	415
		2	324
		3	253
		4	289
		5	272
2	20-3-02	1	179
		2	251
		3	251
		4	393
		5	312
3	21-3-02	1	353
		2	292
		3	375
		4	375
		5	234
4	22-3-02	1	197
		2	127
		3	215
		4	270
		5	110
7	25-3-02	1	77
		2	126
		3	127
		4	61
		5	110
8	26-3-02	1	61
		2	46
		3	110
		4	109
		5	15
9	27-3-02	1	77
		2	160
		3	61
		4	61
		5	127
10	28-3-02	1	30
		2	77
		3	61
		4	61
		5	30
11	29-3-02	1	61
		2	15
		3	30
		4	46
		5	46
16	3-4-02	1	61
		2	46
		3	30
		4	30
		5	46

Table 9.9 **Short-term stability study of batch *Enterococcus faecium* LWL34-24/07/01 stored at 5 °C. Membrane filtration (1 ml of reconstituted capsule solution) on Slanetz and Bartley agar, ISO 7899-2: 2000**

Day	Date	Unit	Duplicate	Count
0	10-6-02	1	1	61
		1	2	54
		2	1	58
		2	2	53
		3	1	57
		3	2	61
		4	1	54
		4	2	60
		5	1	58
		5	2	56
1	11-6-02	1	1	43
		1	2	68
		2	1	51
		2	2	41
		3	1	56
		3	2	58
		4	1	49
		4	2	50
		5	1	41
		5	2	42
16	26-6-02	1	1	61
		1	2	44
		2	1	65
		2	2	62
		3	1	47
		3	2	47
		4	1	57
		4	2	54
		5	1	59
		5	2	47
28	8-7-02	1	1	56
		1	2	58
		2	1	46
		2	2	69
		3	1	56
		3	2	64
		4	1	55
		4	2	47
		5	1	45
		5	2	56
70	19-8-02	1	1	50
		1	2	50
		2	1	51
		2	2	53
		3	1	50
		3	2	46
		4	1	59
		4	2	68
		5	1	53
		5	2	45
98	16-9-02	1	1	59
		1	2	37
		2	1	52
		2	2	47
		3	1	38
		3	2	36
		4	1	47
		4	2	45
		5	1	60
		5	2	51
127	15-10-02	1	1	60
		1	2	67
		2	1	58
		2	2	64
		3	1	56
		3	2	58
		4	1	68
		4	2	51
		5	1	45
		5	2	45
156	13-11-02	1	1	79
		1	2	53
		2	1	70
		2	2	55
		3	1	47
		3	2	50
		4	1	65
		4	2	78
		5	1	58
		5	2	66
182	9-12-02	1	1	51
		1	2	55
		2	1	59
		2	2	66
		3	1	63
		3	2	61
		4	1	69
		4	2	71
		5	1	52
		5	2	57

Table 9.10 **Short-term stability study of batch *Enterococcus faecium* LWL34-24/07/01 stored at 22 °C. Membrane filtration (1 ml of reconstituted capsule solution) on Slanetz and Bartley agar, ISO 7899-2: 2000**

Day	Date	Unit	Duplicate	Count
0	10-6-02	1	1	61
		1	2	54
		2	1	58
		2	2	53
		3	1	57
		3	2	61
		4	1	54
		4	2	60
		5	1	58
		5	2	56
1	11-6-02	1	1	57
		1	2	50
		2	1	45
		2	2	58
		3	1	53
		3	2	44
		4	1	53
		4	2	59
		5	1	59
		5	2	55
7	17-6-02	1	1	42
		1	2	51
		2	1	58
		2	2	50
		3	1	55
		3	2	47
		4	1	54
		4	2	52
		5	1	67
		5	2	51
16	26-6-02	1	1	44
		1	2	56
		2	1	59
		2	2	57
		3	1	60
		3	2	50
		4	1	46
		4	2	36
		5	1	56
		5	2	51
21	1-7-02	1	1	56
		1	2	40
		2	1	52
		2	2	55
		3	1	35
		3	2	40
		4	1	55
		4	2	56
		5	1	42
		5	2	37
28	8-7-02	1	1	64
		1	2	47
		2	1	52
		2	2	58
		3	1	53
		3	2	55
		4	1	50
		4	2	43
		5	1	46
		5	2	41
70	19-8-02	1	1	52
		1	2	54
		2	1	56
		2	2	58
		3	1	52
		3	2	56
		4	1	53
		4	2	68
		5	1	45
		5	2	53
84	2-9-02	1	1	70
		1	2	63
		2	1	63
		2	2	66
		3	1	48
		3	2	58
		4	1	60
		4	2	43
		5	1	49
		5	2	48

Table 9.11 **Short-term stability study of batch *Enterococcus faecium* LWL34-24/07/01 stored at 30 °C. Membrane filtration (1 ml of reconstituted capsule solution) on Slanetz and Bartley agar, ISO 7899-2: 2000**

Day	Date	Unit	Duplicate	Count
0	10-6-02	1	1	61
		1	2	54
		2	1	58
		2	2	53
		3	1	57
		3	2	61
		4	1	54
		4	2	60
		5	1	58
		5	2	56
1	11-6-02	1	1	61
		1	2	47
		2	1	51
		2	2	52
		3	1	53
		3	2	41
		4	1	52
		4	2	42
		5	1	71
		5	2	49
4	14-6-02	1	1	50
		1	2	48
		2	1	60
		2	2	45
		3	1	40
		3	2	43
		4	1	42
		4	2	37
		5	1	59
		5	2	61
7	17-6-02	1	1	48
		1	2	42
		2	1	47
		2	2	51
		3	1	37
		3	2	45
		4	1	58
		4	2	52
		5	1	34
		5	2	48
10	20-6-02	1	1	48
		1	2	67
		2	1	50
		2	2	41
		3	1	48
		3	2	47
		4	1	45
		4	2	36
		5	1	41
		5	2	32
14	24-6-02	1	1	59
		1	2	58
		2	1	40
		2	2	52
		3	1	61
		3	2	47
		4	1	46
		4	2	44
		5	1	49
		5	2	45
17	27-6-02	1	1	32
		1	2	43
		2	1	61
		2	2	53
		3	1	41
		3	2	55
		4	1	53
		4	2	45
		5	1	59
		5	2	51
24	4-7-02	1	1	50
		1	2	41
		2	1	55
		2	2	51
		3	1	52
		3	2	45
		4	1	63
		4	2	66
		5	1	53
		5	2	44

Table 9.12 **Short-term stability study of batch *Enterococcus faecium* LWL34-24/07/01 stored at 36 °C. Membrane filtration (1 ml of reconstituted capsule solution) on Slanetz and Bartley agar, ISO 7899-2: 2000**

Day	Date	Unit	Duplicate	Count
0	10-6-02	1	1	61
		1	2	54
		2	1	58
		2	2	53
		3	1	57
		3	2	61
		4	1	54
		4	2	60
		5	1	58
		5	2	56
1	11-6-02	1	1	58
		1	2	41
		2	1	59
		2	2	43
		3	1	57
		3	2	54
		4	1	36
		4	2	64
		5	1	36
		5	2	36
2	12-6-02	1	1	41
		1	2	43
		2	1	57
		2	2	72
		3	1	56
		3	2	49
		4	1	55
		4	2	61
		5	1	55
		5	2	54
3	13-6-02	1	1	45
		1	2	48
		2	1	60
		2	2	44
		3	1	56
		3	2	51
		4	1	33
		4	2	37
		5	1	51
		5	2	38
4	14-6-02	1	1	57
		1	2	45
		2	1	50
		2	2	51
		3	1	47
		3	2	48
		4	1	65
		4	2	50
		5	1	58
		5	2	55
7	17-6-02	1	1	46
		1	2	48
		2	1	44
		2	2	53
		3	1	57
		3	2	50
		4	1	43
		4	2	47
		5	1	43
		5	2	43
9	19-6-02	1	1	52
		1	2	47
		2	1	56
		2	2	48
		3	1	45
		3	2	50
		4	1	45
		4	2	44
		5	1	37
		5	2	40
11	21-6-02	1	1	55
		1	2	49
		2	1	54
		2	2	45
		3	1	38
		3	2	54
		4	1	51
		4	2	37
		5	1	39
		5	2	49
14	24-6-02	1	1	44
		1	2	58
		2	1	51
		2	2	51
		3	1	52
		3	2	39
		4	1	44
		4	2	61
		5	1	63
		5	2	45
16	26-6-02	1	1	66
		1	2	42
		2	1	44
		2	2	48
		3	1	38
		3	2	49
		4	1	49
		4	2	42
		5	1	49
		5	2	58
18	28-6-02	1	1	51
		1	2	51
		2	1	35
		2	2	57
		3	1	49
		3	2	51
		4	1	56
		4	2	50
		5	1	36
		5	2	35

Table 9.13 **Short-term stability study of batch *Enterococcus faecium* LWL34-24/07/01 stored at 5 °C. Pour plate (1 ml of reconstituted capsule solution) in Yeast Extract agar agar, incubated at 22 °C, ISO 6222: 1999**

Day	Date	Unit	Duplicate	Count
0	10-6-02	1	1	63
		1	2	63
		2	1	58
		2	2	57
		3	1	59
		3	2	71
		4	1	60
		4	2	61
		5	1	57
		5	2	61
1	11-6-02	1	1	66
		1	2	63
		2	1	54
		2	2	63
		3	1	62
		3	2	64
		4	1	55
		4	2	68
		5	1	63
		5	2	60
16	26-6-02	1	1	52
		1	2	49
		2	1	61
		2	2	58
		3	1	55
		3	2	65
		4	1	59
		4	2	60
		5	1	61
		5	2	61
28	8-7-02	1	1	67
		1	2	84
		2	1	82
		2	2	79
		3	1	61
		3	2	64
		4	1	76
		4	2	61
		5	1	64
		5	2	66
70	19-8-02	1	1	51
		1	2	64
		2	1	59
		2	2	48
		3	1	59
		3	2	72
		4	1	63
		4	2	54
		5	1	62
		5	2	65
98	16-9-02	1	1	52
		1	2	54
		2	1	60
		2	2	50
		3	1	69
		3	2	55
		4	1	66
		4	2	68
		5	1	57
		5	2	55
127	15-10-02	1	1	56
		1	2	57
		2	1	65
		2	2	66
		3	1	69
		3	2	51
		4	1	54
		4	2	60
		5	1	51
		5	2	53
156	13-11-02	1	1	70
		1	2	65
		2	1	74
		2	2	58
		3	1	74
		3	2	75
		4	1	85
		4	2	57
		5	1	72
		5	2	81
182	9-12-02	1	1	71
		1	2	86
		2	1	79
		2	2	69
		3	1	75
		3	2	63
		4	1	74
		4	2	75
		5	1	64
		5	2	69

Table 9.14 **Short-term stability study of batch *Enterococcus faecium* LWL34-24/07/01 stored at 22 °C. Pour plate (1 ml of reconstituted capsule solution) in Yeast Extract agar agar, incubated at 22 °C, ISO 6222: 1999**

Day	Date	Unit	Duplicate	Count
0	10-6-02	1	1	63
		1	2	63
		2	1	58
		2	2	57
		3	1	59
		3	2	71
		4	1	60
		4	2	61
		5	1	57
		5	2	61
1	11-6-02	1	1	51
		1	2	61
		2	1	66
		2	2	81
		3	1	67
		3	2	55
		4	1	65
		4	2	65
		5	1	67
		5	2	62
7	17-6-02	1	1	69
		1	2	64
		2	1	71
		2	2	63
		3	1	80
		3	2	52
		4	1	52
		4	2	59
		5	1	60
		5	2	59
16	26-6-02	1	1	59
		1	2	65
		2	1	71
		2	2	65
		3	1	82
		3	2	62
		4	1	75
		4	2	49
		5	1	50
		5	2	37
21	1-7-02	1	1	48
		1	2	61
		2	1	63
		2	2	59
		3	1	53
		3	2	45
		4	1	64
		4	2	61
		5	1	57
		5	2	63
28	8-7-02	1	1	68
		1	2	62
		2	1	62
		2	2	62
		3	1	75
		3	2	78
		4	1	69
		4	2	75
		5	1	69
		5	2	66
70	19-8-02	1	1	72
		1	2	58
		2	1	78
		2	2	61
		3	1	63
		3	2	66
		4	1	56
		4	2	55
		5	1	55
		5	2	63
84	2-9-02	1	1	73
		1	2	71
		2	1	70
		2	2	61
		3	1	72
		3	2	70
		4	1	64
		4	2	65
		5	1	69
		5	2	67

Table 9.15 **Short-term stability study of batch *Enterococcus faecium* LWL34-24/07/01 stored at 30 °C. Pour plate (1 ml of reconstituted capsule solution) in Yeast Extract agar agar, incubated at 22 °C, ISO 6222: 1999**

Day	Date	Unit	Duplicate	Count
0	10-6-02	1	1	63
		1	2	63
		2	1	58
		2	2	57
		3	1	59
		3	2	71
		4	1	60
		4	2	61
		5	1	57
		5	2	61
1	11-6-02	1	1	48
		1	2	46
		2	1	53
		2	2	59
		3	1	59
		3	2	62
		4	1	64
		4	2	63
		5	1	62
		5	2	63
4	14-6-02	1	1	55
		1	2	51
		2	1	60
		2	2	73
		3	1	58
		3	2	70
		4	1	58
		4	2	56
		5	1	58
		5	2	70
7	17-6-02	1	1	61
		1	2	54
		2	1	66
		2	2	52
		3	1	66
		3	2	48
		4	1	50
		4	2	52
		5	1	49
		5	2	55
10	20-6-02	1	1	64
		1	2	56
		2	1	56
		2	2	51
		3	1	46
		3	2	66
		4	1	52
		4	2	56
		5	1	50
		5	2	56
14	24-6-02	1	1	54
		1	2	51
		2	1	58
		2	2	48
		3	1	62
		3	2	57
		4	1	51
		4	2	43
		5	1	70
		5	2	53
17	27-6-02	1	1	45
		1	2	41
		2	1	76
		2	2	70
		3	1	49
		3	2	46
		4	1	51
		4	2	53
		5	1	59
		5	2	50
24	4-7-02	1	1	62
		1	2	68
		2	1	58
		2	2	80
		3	1	78
		3	2	68
		4	1	51
		4	2	67
		5	1	68
		5	2	56

Table 9.16 **Short-term stability study of batch *Enterococcus faecium* LWL34-24/07/01 stored at 36 °C. Pour plate (1 ml of reconstituted capsule solution) in Yeast Extract agar agar, incubated at 22 °C, ISO 6222: 1999**

Day	Date	Unit	Duplicate	Count
0	10-6-02	1	1	63
		1	2	63
		2	1	58
		2	2	57
		3	1	59
		3	2	71
		4	1	60
		4	2	61
		5	1	57
		5	2	61
1	11-6-02	1	1	75
		1	2	56
		2	1	45
		2	2	48
		3	1	70
		3	2	77
		4	1	62
		4	2	60
		5	1	78
		5	2	67
2	12-6-02	1	1	44
		1	2	49
		2	1	53
		2	2	66
		3	1	50
		3	2	46
		4	1	54
		4	2	40
		5	1	48
		5	2	57
3	13-6-02	1	1	44
		1	2	58
		2	1	54
		2	2	49
		3	1	61
		3	2	60
		4	1	63
		4	2	46
		5	1	44
		5	2	48
4	14-6-02	1	1	43
		1	2	62
		2	1	74
		2	2	55
		3	1	59
		3	2	48
		4	1	43
		4	2	51
		5	1	59
		5	2	58
7	17-6-02	1	1	60
		1	2	51
		2	1	48
		2	2	53
		3	1	59
		3	2	53
		4	1	57
		4	2	56
		5	1	47
		5	2	53
9	19-6-02	1	1	53
		1	2	46
		2	1	47
		2	2	56
		3	1	67
		3	2	67
		4	1	53
		4	2	73
		5	1	61
		5	2	46
11	21-6-02	1	1	52
		1	2	58
		2	1	47
		2	2	46
		3	1	45
		3	2	56
		4	1	49
		4	2	65
		5	1	75
		5	2	55
14	24-6-02	1	1	55
		1	2	42
		2	1	62
		2	2	62
		3	1	67
		3	2	50
		4	1	54
		4	2	60
		5	1	58
		5	2	57
16	26-6-02	1	1	61
		1	2	54
		2	1	44
		2	2	56
		3	1	51
		3	2	50
		4	1	41
		4	2	50
		5	1	67
		5	2	60
18	28-6-02	1	1	51
		1	2	51
		2	1	43
		2	2	58
		3	1	61
		3	2	69
		4	1	44
		4	2	47
		5	1	49
		5	2	55

Table 9.17 **Short-term stability study of batch *Enterococcus faecium* LWL34-24/07/01 stored at 5 °C. Pour plate (1 ml of reconstituted capsule solution) in Yeast Extract agar agar, incubated at 36 °C, ISO 6222: 1999**

Day	Date	Unit	Duplicate	Count
0	03-12-01	1	1	42
		1	2	52
		2	1	58
		2	2	58
		3	1	52
		3	2	47
		4	1	35
		4	2	62
		5	1	66
		5	2	53
1	04-12-01	1	1	65
		1	2	68
		2	1	58
		2	2	58
		3	1	54
		3	2	49
		4	1	57
		4	2	52
		5	1	48
		5	2	54
14	17-12-01	1	1	47
		1	2	54
		2	1	74
		2	2	68
		3	1	50
		3	2	56
		4	1	64
		4	2	54
		5	1	52
		5	2	62
42	14-01-02	1	1	47
		1	2	44
		2	1	57
		2	2	58
		3	1	60
		3	2	50
		4	1	52
		4	2	55
		5	1	59
		5	2	50
56	28-01-02	1	1	55
		1	2	54
		2	1	62
		2	2	51
		3	1	55
		3	2	41
		4	1	55
		4	2	59
		5	1	45
		5	2	56
70	11-02-02	1	1	55
		1	2	49
		2	1	57
		2	2	58
		3	1	63
		3	2	44
		4	1	60
		4	2	65
		5	1	45
		5	2	52
98	11-03-02	1	1	55
		1	2	57
		2	1	60
		2	2	53
		3	1	54
		3	2	52
		4	1	53
		4	2	53
		5	1	52
		5	2	52
126	08-04-02	1	1	61
		1	2	59
		2	1	48
		2	2	73
		3	1	58
		3	2	65
		4	1	62
		4	2	66
		5	1	52
		5	2	71
154	06-05-02	1	1	64
		1	2	59
		2	1	62
		2	2	50
		3	1	77
		3	2	64
		4	1	68
		4	2	70
		5	1	64
		5	2	62
182	03-06-02	1	1	64
		1	2	65
		2	1	44
		2	2	58
		3	1	57
		3	2	50
		4	1	50
		4	2	53
		5	1	61
		5	2	54

Table 9.18 **Short-term stability study of batch *Enterococcus faecium* LWL34-24/07/01 stored at 22 °C. Pour plate (1 ml of reconstituted capsule solution) in Yeast Extract agar agar, incubated at 36 °C, ISO 6222: 1999**

Day	Date	Unit	Duplicate	Count
0	03-12-01	1	1	42
		1	2	52
		2	1	58
		2	2	58
		3	1	52
		3	2	47
		4	1	35
		4	2	62
		5	1	66
		5	2	53
1	04-12-01	1	1	56
		1	2	59
		2	1	37
		2	2	45
		3	1	42
		3	2	38
		4	1	59
		4	2	56
		5	1	45
		5	2	40
7	10-12-01	1	1	54
		1	2	51
		2	1	55
		2	2	60
		3	1	50
		3	2	64
		4	1	53
		4	2	59
		5	1	56
		5	2	54
14	17-12-01	1	1	55
		1	2	60
		2	1	53
		2	2	50
		3	1	65
		3	2	66
		4	1	39
		4	2	37
		5	1	53
		5	2	44
37	09-01-02	1	1	70
		1	2	55
		2	1	82
		2	2	59
		3	1	66
		3	2	57
		4	1	52
		4	2	69
		5	1	58
		5	2	78
42	14-01-02	1	1	65
		1	2	58
		2	1	63
		2	2	61
		3	1	59
		3	2	61
		4	1	53
		4	2	43
		5	1	47
		5	2	42
56	28-01-02	1	1	35
		1	2	54
		2	1	45
		2	2	47
		3	1	44
		3	2	43
		4	1	53
		4	2	49
		5	1	51
		5	2	49
70	11-02-02	1	1	44
		1	2	40
		2	1	47
		2	2	43
		3	1	40
		3	2	42
		4	1	44
		4	2	39
		5	1	40
		5	2	46

Table 9.19 **Short-term stability study of batch *Enterococcus faecium* LWL34-24/07/01 stored at 30 °C. Pour plate (1 ml of reconstituted capsule solution) in Yeast Extract agar agar, incubated at 36 °C, ISO 6222: 1999**

Day	Date	Unit	Duplicate	Count
0	03-12-01	1	1	42
		1	2	52
		2	1	58
		2	2	58
		3	1	52
		3	2	47
		4	1	35
		4	2	62
		5	1	66
		5	2	53
1	04-12-01	1	1	59
		1	2	51
		2	1	67
		2	2	47
		3	1	64
		3	2	54
		4	1	56
		4	2	60
		5	1	57
		5	2	53
4	07-12-01	1	1	55
		1	2	42
		2	1	51
		2	2	45
		3	1	59
		3	2	49
		4	1	34
		4	2	56
		5	1	59
		5	2	47
7	10-12-01	1	1	51
		1	2	59
		2	1	52
		2	2	52
		3	1	61
		3	2	55
		4	1	51
		4	2	52
		5	1	52
		5	2	51
10	13-12-01	1	1	44
		1	2	56
		2	1	46
		2	2	44
		3	1	62
		3	2	45
		4	1	49
		4	2	37
		5	1	45
		5	2	49
17	17-12-01	1	1	61
		1	2	44
		2	1	45
		2	2	50
		3	1	40
		3	2	52
		4	1	42
		4	2	61
		5	1	49
		5	2	52
20	20-12-01	1	1	59
		1	2	62
		2	1	56
		2	2	69
		3	1	43
		3	2	60
		4	1	49
		4	2	43
		5	1	60
		5	2	51
41	10-01-02	1	1	62
		1	2	78
		2	1	46
		2	2	52
		3	1	67
		3	2	78
		4	1	66
		4	2	63
		5	1	59
		5	2	65
48	17-01-02	1	1	45
		1	2	48
		2	1	52
		2	2	62
		3	1	45
		3	2	53
		4	1	49
		4	2	55
		5	1	46
		5	2	42

Table 9.20 **Short-term stability study of batch *Enterococcus faecium* LWL34-24/07/01 stored at 36 °C. Pour plate (1 ml of reconstituted capsule solution) in Yeast Extract agar agar, incubated at 36 °C, ISO 6222: 1999**

Day	Date	Unit	Duplicate	Count
0	03-12-01	1	1	42
		1	2	52
		2	1	58
		2	2	58
		3	1	52
		3	2	47
		4	1	35
		4	2	62
		5	1	66
		5	2	53
1	04-12-01	1	1	45
		1	2	55
		2	1	67
		2	2	59
		3	1	56
		3	2	57
		4	1	56
		4	2	63
		5	1	59
		5	2	52
2	05-12-01	1	1	56
		1	2	58
		2	1	50
		2	2	63
		3	1	55
		3	2	51
		4	1	50
		4	2	44
		5	1	56
		5	2	41
3	06-12-01	1	1	61
		1	2	45
		2	1	46
		2	2	52
		3	1	36
		3	2	53
		4	1	56
		4	2	64
		5	1	59
		5	2	49
4	07-12-01	1	1	50
		1	2	59
		2	1	65
		2	2	47
		3	1	47
		3	2	51
		4	1	61
		4	2	50
		5	1	41
		5	2	50
7	10-12-01	1	1	49
		1	2	45
		2	1	56
		2	2	58
		3	1	56
		3	2	64
		4	1	64
		4	2	60
		5	1	61
		5	2	58
9	12-12-01	1	1	47
		1	2	47
		2	1	47
		2	2	53
		3	1	56
		3	2	57
		4	1	50
		4	2	63
		5	1	47
		5	2	58
11	14-12-01	1	1	54
		1	2	60
		2	1	58
		2	2	71
		3	1	52
		3	2	56
		4	1	56
		4	2	53
		5	1	49
		5	2	54
14	17-12-01	1	1	49
		1	2	55
		2	1	59
		2	2	62
		3	1	56
		3	2	50
		4	1	60
		4	2	46
		5	1	50
		5	2	47
16	19-12-01	1	1	48
		1	2	50
		2	1	44
		2	2	64
		3	1	49
		3	2	47
		4	1	45
		4	2	45
		5	1	50
		5	2	47
18	21-12-01	1	1	47
		1	2	55
		2	1	52
		2	2	45
		3	1	48
		3	2	45
		4	1	49
		4	2	43
		5	1	60
		5	2	51

Table 9.21 **Short-term stability study of batch *Enterococcus faecium* LWL34-24/07/01, stored at 5 °C. MPN (3 ml of reconstituted capsule solution) on microtitre plates, ISO 7899-1: 1998a. MPN/ 100 ml are given (only singular counts were made).**

Day	Date	Unit	Count
0	10-06-02	1	287
		2	94
		3	161
		4	94
		5	312
1	11-06-02	1	251
		2	144
		3	195
		4	94
		5	197
16	26-06-02	1	215
		2	309
		3	177
		4	179
		5	144
28	08-07-02	1	144
		2	94
		3	144
		4	215
		5	234
70	19-08-2002	1	270
		2	350
		3	161
		4	126
		5	234
98	16-09-02	1	179
		2	232
		3	215
		4	292
		5	270
127	15-10-02	1	232
		2	144
		3	234
		4	161
		5	179
156	13-11-02	1	253
		2	253
		3	253
		4	253
		5	197
182	09-12-02	1	197
		2	213
		3	179
		4	195
		5	213

Table 9.22 **Short-term stability study of batch *Enterococcus faecium* LWL34-24/07/01, stored at 22 °C. MPN (3 ml of reconstituted capsule solution) on microtitre plates, ISO 7899-1: 1998a. MPN/ 100 ml are given (only singular counts were made).**

Day	Date	Unit	Count
0	10-06-02	1	287
		2	94
		3	161
		4	94
		5	312
1	11-06-02	1	213
		2	197
		3	143
		4	143
		5	197
7	17-06-02	1	197
		2	415
		3	272
		4	253
		5	197
16	26-06-02	1	161
		2	144
		3	232
		4	197
		5	197
21	01-07-02	1	213
		2	249
		3	161
		4	215
		5	213
28	08-07-02	1	251
		2	127
		3	234
		4	253
		5	232
70	19-08-02	1	194
		2	143
		3	230
		4	197
		5	176
84	02-09-02	1	253
		2	309
		3	253
		4	197
		5	197

Table 9.23 **Short-term stability study of batch *Enterococcus faecium* LWL34-24/07/01, stored at 30 °C. MPN (3 ml of reconstituted capsule solution) on microtitre plates, ISO 7899-1: 1998a. MPN/ 100 ml are given (only singular counts were made).**

Day	Date	Unit	Count
0	10-06-02	1	287
		2	94
		3	161
		4	94
		5	312
1	11-06-02	1	251
		2	126
		3	249
		4	177
		5	289
4	14-06-02	1	179
		2	251
		3	249
		4	234
		5	195
7	17-06-02	1	268
		2	353
		3	161
		4	253
		5	232
10	20-06-02	1	197
		2	179
		3	215
		4	94
		5	197
14	24-06-02	1	197
		2	330
		3	160
		4	77
		5	232
17	27-06-02	1	127
		2	110
		3	215
		4	195
		5	142
24	04-07-02	1	309
		2	213
		3	161
		4	177
		5	249

Table 9.24 **Short-term stability study of batch *Enterococcus faecium* LWL34-24/07/01, stored at 36 °C. MPN (3 ml of reconstituted capsule solution) on microtitre plates, ISO 7899-1: 1998a. MPN/ 100 ml are given (only singular counts were made).**

Day	Date	Unit	Count
0	10-06-02	1	287
		2	94
		3	161
		4	94
		5	312
1	11-06-02	1	215
		2	195
		3	142
		4	272
		5	272
2	12-06-02	1	161
		2	251
		3	109
		4	332
		5	144
3	13-06-02	1	197
		2	270
		3	144
		4	232
		5	270
4	14-06-02	1	110
		2	215
		3	127
		4	144
		5	350
7	17-6-02	1	289
		2	287
		3	161
		4	161
		5	215
9	19-6-02	1	161
		2	249
		3	289
		4	110
		5	179
11	21-06-02	1	161
		2	272
		3	177
		4	161
		5	197
14	24-06-02	1	249
		2	393
		3	177
		4	251
		5	213
16	26-06-02	1	77
		2	415
		3	127
		4	215
		5	179
18	28-06-02	1	212
		2	177
		3	144
		4	46
		5	127

Table 9.25 **Short-term stability study of batch *Clostridium perfringens* LWL3501-24/10/01 stored at 5 °C. Membrane filtration (1 ml of reconstituted capsule solution) on Tryptose Cycloserine agar, ISO 6461-2: 2001**

Day	Date	Unit	Duplicate	Count
0	14-01-02	1	1	63
		1	2	48
		2	1	70
		2	2	74
		3	1	61
		3	2	62
		4	1	57
		4	2	61
		5	1	60
		5	2	67
1	15-01-02	1	1	39
		1	2	39
		2	1	41
		2	2	59
		3	1	46
		3	2	55
		4	1	55
		4	2	40
		5	1	62
		5	2	50
14	28-01-02	1	1	40
		1	2	48
		2	1	48
		2	2	51
		3	1	61
		3	2	45
		4	1	37
		4	2	45
		5	1	56
		5	2	47
28	11-02-02	1	1	66
		1	2	81
		2	1	56
		2	2	74
		3	1	58
		3	2	41
		4	1	59
		4	2	71
		5	1	72
		5	2	67
56	11-03-02	1	1	47
		1	2	42
		2	1	43
		2	2	40
		3	1	50
		3	2	38
		4	1	49
		4	2	31
		5	1	49
		5	2	35
70	25-03-02	1	1	63
		1	2	48
		2	1	50
		2	2	55
		3	1	53
		3	2	48
		4	1	52
		4	2	56
		5	1	54
		5	2	49
98	22-04-02	1	1	73
		1	2	67
		2	1	84
		2	2	68
		3	1	71
		3	2	61
		4	1	65
		4	2	77
		5	1	70
		5	2	64
127	21-05-02	1	1	75
		1	2	73
		2	1	72
		2	2	69
		3	1	84
		3	2	88
		4	1	82
		4	2	66
		5	1	70
		5	2	72
154	17-06-02	1	1	58
		1	2	64
		2	1	64
		2	2	57
		3	1	61
		3	2	67
		4	1	54
		4	2	58
		5	1	59
		5	2	65

Table 9.26 **Short-term stability study of batch *Clostridium perfringens* LWL3501-24/10/01 stored at 22 °C. Membrane filtration (1 ml of reconstituted capsule solution) on Tryptose Cycloserine agar, ISO 6461-2: 2001**

Day	Date	Unit	Duplicate	Count
0	14-01-02	1	1	63
		1	2	48
		2	1	70
		2	2	74
		3	1	61
		3	2	62
		4	1	57
		4	2	61
		5	1	60
		5	2	67
1	15-01-02	1	1	55
		1	2	55
		2	1	48
		2	2	62
		3	1	67
		3	2	70
		4	1	58
		4	2	55
		5	1	44
		5	2	50
7	21-01-02	1	1	36
		1	2	41
		2	1	66
		2	2	57
		3	1	50
		3	2	51
		4	1	36
		4	2	51
		5	1	67
		5	2	55
14	28-01-01	1	1	71
		1	2	72
		2	1	78
		2	2	71
		3	1	58
		3	2	66
		4	1	61
		4	2	70
		5	1	47
		5	2	65
21	04-02-01	1	1	63
		1	2	67
		2	1	53
		2	2	58
		3	1	63
		3	2	65
		4	1	60
		4	2	65
		5	1	69
		5	2	82
28	11-02-02	1	1	58
		1	2	63
		2	1	45
		2	2	49
		3	1	63
		3	2	54
		4	1	67
		4	2	66
		5	1	63
		5	2	43
35	18-02-02	1	1	52
		1	2	38
		2	1	42
		2	2	49
		3	1	45
		3	2	35
		4	1	46
		4	2	51
		5	1	43
		5	2	62
56	11-03-02	1	1	38
		1	2	41
		2	1	52
		2	2	38
		3	1	51
		3	2	49
		4	1	36
		4	2	30
		5	1	38
		5	2	42
70	25-03-02	1	1	55
		1	2	46
		2	1	60
		2	2	49
		3	1	68
		3	2	51
		4	1	45
		4	2	55
		5	1	46
		5	2	57
84	08-04-02	1	1	70
		1	2	69
		2	1	67
		2	2	56
		3	1	69
		3	2	76
		4	1	59
		4	2	69
		5	1	71
		5	2	79

Table 9.27 **Short-term stability study of batch *Clostridium perfringens* LWL3501-24/10/01 stored at 30 °C. Membrane filtration (1 ml of reconstituted capsule solution) on Tryptose Cycloserine agar, ISO 6461-2: 2001**

Day	Date	Unit	Duplicate	Count
0	14-01-02	1	1	63
		1	2	48
		2	1	70
		2	2	74
		3	1	61
		3	2	62
		4	1	57
		4	2	61
		5	1	60
		5	2	67
1	15-01-02	1	1	65
		1	2	72
		2	1	56
		2	2	53
		3	1	61
		3	2	67
		4	1	59
		4	2	57
		5	1	78
		5	2	61
4	18-01-02	1	1	76
		1	2	69
		2	1	56
		2	2	64
		3	1	66
		3	2	75
		4	1	59
		4	2	64
		5	1	63
		5	2	64
7	21-01-02	1	1	47
		1	2	48
		2	1	42
		2	2	42
		3	1	58
		3	2	47
		4	1	43
		4	2	60
		5	1	70
		5	2	45
10	24-01-02	1	1	52
		1	2	71
		2	1	72
		2	2	73
		3	1	48
		3	2	66
		4	1	54
		4	2	61
		5	1	52
		5	2	56
14	28-01-02	1	1	69
		1	2	72
		2	1	72
		2	2	75
		3	1	58
		3	2	57
		4	1	62
		4	2	52
		5	1	63
		5	2	65
17	31-01-02	1	1	68
		1	2	73
		2	1	62
		2	2	80
		3	1	54
		3	2	79
		4	1	69
		4	2	60
		5	1	80
		5	2	64
24	07-02-02	1	1	54
		1	2	42
		2	1	35
		2	2	45
		3	1	50
		3	2	45
		4	1	50
		4	2	42
		5	1	55
		5	2	42
31	14-02-02	1	1	69
		1	2	55
		2	1	58
		2	2	69
		3	1	56
		3	2	51
		4	1	74
		4	2	72
		5	1	53
		5	2	48
38	21-02-02	1	1	43
		1	2	50
		2	1	58
		2	2	64
		3	1	46
		3	2	61
		4	1	58
		4	2	58
		5	1	49
		5	2	41
51	06-03-02	1	1	46
		1	2	53
		2	1	51
		2	2	62
		3	1	68
		3	2	59
		4	1	41
		4	2	68
		5	1	54
		5	2	55

Table 9.28 **Short-term stability study of batch *Clostridium perfringens* LWL3501-24/10/01 stored at 36 °C. Membrane filtration (1 ml of reconstituted capsule solution) on Tryptose Cycloserine agar, ISO 6461-2: 2001**

Day	Date	Unit	Duplicate	Count
0	14-01-02	1	1	63
		1	2	48
		2	1	70
		2	2	74
		3	1	61
		3	2	62
		4	1	57
		4	2	61
		5	1	60
		5	2	67
1	15-01-02	1	1	51
		1	2	49
		2	1	50
		2	2	56
		3	1	50
		3	2	49
		4	1	48
		4	2	50
		5	1	56
		5	2	79
2	16-01-02	1	1	53
		1	2	52
		2	1	36
		2	2	47
		3	1	70
		3	2	62
		4	1	51
		4	2	67
		5	1	62
		5	2	61
3	17-01-02	1	1	53
		1	2	60
		2	1	60
		2	2	60
		3	1	55
		3	2	54
		4	1	55
		4	2	49
		5	1	51
		5	2	50
4	18-01-02	1	1	62
		1	2	68
		2	1	73
		2	2	63
		3	1	62
		3	2	80
		4	1	62
		4	2	63
		5	1	79
		5	2	78
7	21-01-02	1	1	58
		1	2	59
		2	1	50
		2	2	52
		3	1	47
		3	2	50
		4	1	51
		4	2	54
		5	1	48
		5	2	52
9	23-01-02	1	1	50
		1	2	45
		2	1	63
		2	2	47
		3	1	49
		3	2	47
		4	1	52
		4	2	44
		5	1	60
		5	2	76
11	25-01-02	1	1	63
		1	2	74
		2	1	59
		2	2	58
		3	1	76
		3	2	74
		4	1	54
		4	2	62
		5	1	65
		5	2	60
14	28-01-02	1	1	58
		1	2	61
		2	1	63
		2	2	61
		3	1	59
		3	2	57
		4	1	48
		4	2	51
		5	1	72
		5	2	80
16	30-01-02	1	1	53
		1	2	58
		2	1	38
		2	2	41
		3	1	52
		3	2	68
		4	1	61
		4	2	42
		5	1	56
		5	2	52
18	01-02-02	1	1	60
		1	2	73
		2	1	62
		2	2	67
		3	1	67
		3	2	52
		4	1	58
		4	2	54
		5	1	58
		5	2	72