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Certification of a reference material with *Salmonella enteritidis* (NCTC 12694) at a level of 5 colony forming units on nutrient agar and 4 colony forming units on xylose lysine deoxycholate agar, IRMM-352

L. De Baets, P. van Iwaarden, W. Bremser, N. Meeus, W. Philipp, H. Schimmel

EUR 23541 EN - 2008

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EUR 23541 EN
ISBN 978-92-79-10272-1
ISSN 1018-5593
DOI 10.2787/85196

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IRMM information
REFERENCE MATERIALS

Certification of a reference material with *Salmonella enteritidis* (NCTC 12694) at a level of 5 colony forming units on nutrient agar and 4 colony forming units on xylose lysine deoxycholate agar

IRMM-352

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Abstract

This report describes the certification of a reference material (IRMM-352) of *Salmonella enteritidis*. Certified Reference Materials (CRMs) for microbiological analysis are indispensable tools for development and validation of detection methods and for the implementation and support of internal and external quality control in the area of microbiological analysis. Each vial contains one material sphere of *S. enteritidis*. The homogeneity and stability (at 4 °C, -20 °C and -70 °C) of the batch was assessed by monitoring colony forming units (cfu) on nutrient agar (NA) and xylose lysine deoxycholate (XLD) agar of selected vials by colony counting. The material is not stable at 4 °C but no instability was detected when stored at -20 °C for up to 12 months and at -70 °C for up to 54 months. The batch was characterised by six laboratories to determine a certified value of cfu per vial on NA and XLD agar. A quite considerable performance difference of both agars was observed. The certified value is 5 cfu on NA with an expanded uncertainty of 2 and 4 cfu on XLD agar with an expanded uncertainty of 2 using a coverage factor $k = 2$, corresponding to a level of confidence of about 95 %. DNA sequence analysis of the coding region for the *sef*A gene identified the material as *S. enteritidis*.

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ANNEX 1: MICROBIOLOGICAL CRMS AVAILABLE FROM IRMM **35**

Glossary

ANOVA	analysis of variance
BLAST	Basic Local Alignment Search Tool
bp	base pair
c_{dg}	degradation rate
cfu	colony forming unit
CRM	Certified Reference Material
DTCS	dye terminator cycle sequencing
dNTP	deoxynucleotide triphosphates
DNA	deoxyribonucleic acid
f_i	number fraction
gDNA	genomic DNA
i	number of cfu in object class
i_{cert}	certified cfu value
i_{end}	mean cfu value at the end of stability monitoring at -70 °C
i_{est}	mean cfu value at the end of the expected shelf-life
$\bar{i}_{char}$	mean cfu value for characterisation
$\bar{i}_{hom}$	mean cfu value for homogeneity
IRMM	Institute for Reference Materials and Measurements
k	coverage factor
LTS	long term stability
M	batch size
m	sample size
m.p.n.	most probable number
N	total number of vials in the homogeneity study
n	total number of replicates
NA	nutrient agar
NCTC	national collection of type cultures
P	probability of the distribution
PC	post certification
PCR	polymerase chain reaction
p	experimental probability
p_i	experimental probability in object class i
RM	reference material
RSD	relative standard deviation

s	standard deviation
*sef*A	gene encoding *Salmonella enteritidis* fimbrial antigen
STS	short term stability
Δt_{study}	duration of the study
t_{shelf}	expected shelf-life
U	expanded uncertainty
u	standard uncertainty
$\bar{x}$	mean
XLD	xylose lysine deoxycholate

1 Introduction

Certified Reference Materials (CRMs) for microbiological analysis are indispensable tools for development and validation of detection methods and for the implementation and support of internal and external quality control in the area of microbiological analysis. IRMM offers already microbiological RMs with certified colony forming unit (cfu) numbers per matrix unit covering six different food and water micro-organisms (annex 1). Statistical effects, such as Poisson distribution at low cfu numbers, encouraged us to consider alternative approaches for development and certification of reference materials. IRMM currently extends the range of CRMs to more micro-organisms with relevant cfu counts, including *Salmonella enteritidis* at a level of about 5 cfu per material sphere, or new CRMs at a level of 1000 cfu per material sphere for water control laboratories.

This report describes the certification of a batch of 800 vials (IRMM-352) containing *S. enteritidis* at a target level of 5 cfu per material sphere. Homogeneity and stability of the produced batch were analysed at IRMM following IRMM RM Unit procedures applying a quality management system according to ISO Guide 34 [1-4]. Batch characterisation was performed by IRMM and five external laboratories. The procedures used for the batch characterisation as well as for homogeneity and stability testing were colony counting on nutrient agar (NA) and xylose lysine deoxycholate (XLD) agar according to ISO 7218 [5] and ISO 6579 [6], respectively.

Identification of *S. enteritidis* was performed by DNA sequence analysis of the *sef*A gene encoding a fimbrial antigen.

2 Participants

The reference material was developed and produced by BTF Pty Ltd. (North Ryde, AU).

Analysis of homogeneity and stability and additional characterisation of IRMM-352 was performed at IRMM. Participants for batch characterisation measurements were:

- IRMM, Joint Research Centre, European Commission, Geel, BE
- CCFRA Technology Ltd, Gloucestershire, GB
- TNO Quality of Life, Zeist, NL
- Animal Sciences Group, Lelystad, NL
- Institut Pasteur de Lille, Lille, FR
- Food and Consumer Product Safety Authority, Zutphen, NL

3 Processing steps

IRMM-352 was produced at BTF Pty Ltd. (North Ryde, AU) in the BioBall^{TM} format [7]. For the production, a modified flow cytometer was used. The latter allows the sorting and counting of a number of bacterial cells into a single droplet. For the reference material discussed here the target value was set at 5 cells per droplet. In a second step the droplet was collected in a glass vial with liquid nitrogen resulting in a frozen ball. Finally the ball was freeze-dried using a lyoprotectant consisting of a protein-carbohydrate matrix. This lyoprotectant allows a maximal viability of the bacterial cells in the material sphere. Glass vials were closed with a rubber stopper and stored at -70°C until analysis. In total, 800 vials of IRMM-352 were produced.

4 Procedures

4.1 Minimum sample volume

One material sphere is used per assay.

4.2 Colony counting

One material sphere was reconstituted as described in section 10.2. Colony counting was performed on NA according to ISO 7218 [5] and on XLD agar according to ISO 6579 [6]. Plates were incubated at 37 °C for 15 to 22 h.

4.3 PCR

Each reaction mixture for PCR reactions (25 µL total volume) contained PCR buffer (20 mM Tris-HCl (pH 8.4), 50 mM KCl), 1.5 mmol/L $MgCl_2$, 200 µmol/L dNTP, 0.4 µmol/L forward primer, 0.4 µmol/L reverse primer, 1 unit Platinum® *Taq* DNA polymerase. As a template for the PCR reaction part of one bacterial colony was used. PCR primers used are summarised in Table 1. All other reaction components for PCR reactions were provided by Invitrogen (Merelbeke, BE). PCR reactions were performed using the following time programme: denaturation genomic DNA (gDNA) at 94 °C for 5 min; 40 cycles denaturation 94 °C for 60 s, annealing 61 °C for 60 s, extension 72 °C for 90 s; incubation 72 °C for 10 min; hold 4 °C. PCR products were visualised by agarose gel electrophoresis.

Table 1. Primers used for qualitative PCR.

Organism	PCR target, amplicon length	Primer	DNA sequence	Reference
Salmonella enteritidis	*sef*A gene, 310 bp	SEFA2 SEFA4	5'-GCA GCG GTT ACT ATT GCA GC-3' 5'-TGT GAC AGG GAC ATT TAG CG-3'	[8]

4.4 DNA sequencing

PCR products (25 µL), amplified with primers SEFA2 and SEFA4, were purified using the QiaQuick® PCR Purification Kit (Qiagen, Venlo, NL) and cloned into pCR2.1 (Invitrogen, Merelbeke, BE). Plasmid DNA was purified using the QIAprep Spin Miniprep Kit (Qiagen, Venlo, NL). Purified plasmid DNA was used in the dye terminator cycle sequencing (DTCS) reaction, according to the DTCS chemistry protocol [9].

Samples were analysed on a CEQ™ 8000 genetic analysis system (Beckman Coulter, Inc., Fullerton, CA, US), using the following method: denaturation 120 s at 90 °C, injection 15 s at 2.0 kV, separation 85.0 min at 4.2 kV. As a control for the sequencing reaction the CEQ8000

pUC18 plasmid was included on each sequencing plate. Validation of DNA sequencing using the CEQ8000 system is described in detail in ref [10].

5 Homogeneity and characterisation of the batch

The measurand in this certification project is the number of cfu per material sphere. Since cfu is a discrete variable and numbers are small, the commonly accepted CRM strategies for continuous variables which extensively use analysis of variance (ANOVA) are not applicable. Moreover, there is no difference in the procedure used for homogeneity testing and batch characterisation. Therefore, an approach treating homogeneity test results and characterisation results on an equal level was implemented.

5.1 Planning and results of homogeneity study

800 vials were produced and the total number of vials to be used in the homogeneity study (N) was calculated from $\sqrt[3]{800} = 9.28$ [2]. Because of the high relative standard deviation (RSD%) obtained for low cfu values, the number of vials in the homogeneity study was increased to 30 per procedure. For each analysis the sample intake was one material sphere i.e. $n = 1$ measurement per vial. Vials for the homogeneity study were taken from the stock at -70 °C. Cfu values per material sphere were determined by plating and colony counting on NA, according to ISO 7218 [5] and on XLD agar, according to ISO 6579 [6]. Samples were incubated at 37 °C for 15 to 22 h. The results of the homogeneity measurements are summarised in Table 2.

Table 2. Measurement data for the homogeneity study of IRMM-352 (n = 1). Individual data per vial as well as the mean ($\bar{x}$), standard deviation (s) and relative standard deviation (RSD%) are shown for both agars.

NA		XLD agar	
Vial identification number	**Measured value (cfu)**	**Vial identification number**	**Measured value (cfu)**
17	5	7	3
45	4	15	2
66	5	28	2
100	5	108	2
121	5	123	4
148	7	150	1
181	5	185	6
184	8	188	4
232	3	235	4
258	5	257	3
276	4	278	1
295	5	293	3
314	6	317	3
350	5	352	4
382	5	384	6
394	5	396	3
437	4	440	4
444	4	446	6
493	7	494	4
496	5	501	6
533	5	577	4
553	2	581	1
582	6	585	2
605	6	589	1
639	5	607	2
668	5	642	2
680	3	727	5
715	5	733	5
753	3	755	3
761	6	763	5
$\bar{x}$	4.9	$\bar{x}$	3.4
s	1.3	***s***	1.6
RSD%	25	**RSD%**	47

5.2 Planning and results of batch characterisation study

The batch of IRMM-352 was characterised by six laboratories by plating and colony counting of 15 vials on NA, according to ISO 7218 [5] and 15 vials on XLD agar, according to ISO 6579 [6]. Results of these measurements are summarised in Table 3.

Table 3. Measurement data for batch characterisation of IRMM-352. Individual data per vial as well as the mean ($\bar{x}$), standard deviation (*s*) and relative standard deviation (RSD%) are shown for both agars. Suspicious data sets are highlighted in gray.

A. NA

	Measured value (cfu)					
Vial no	**Lab 1**	**Lab 2**	**Lab 3**	**Lab 4**	**Lab 5**	**Lab 6**
1	5	5	2	4	4	2
2	4	3	4	2	5	4
3	5	5	6	5	5	2
4	5	3	7	3	5	4
5	5	7	4	4	2	6
6	7	4	5	3	6	5
7	5	2	4	5	6	6
8	8	4	3	5	3	6
9	3	6	5	2	4	5
10	5	6	5	4	3	4
11	4	3	3	4	8	5
12	5	3	6	5	4	6
13	6	6	5	4	4	5
14	5	4	6	5	5	4
15	5	2	6	5	0	3
$\bar{x}$	5.1	4.2	4.7	4.0	4.3	4.5
s	1.2	1.6	1.4	1.1	1.9	1.4
RSD%	23	37	29	27	44	30

B. XLD

	Measured value (cfu)					
Vial no	**Lab 1**	**Lab 2**	**Lab 3**	**Lab 4**	**Lab 5**	**Lab 6**
1	5	3	3	3	3	3
2	5	6	3	3	3	1
3	1	2	2	3	7	0
4	5	3	4	4	4	1
5	5	1	3	4	4	0
6	2	4	2	2	1	0
7	2	4	3	4	4	3
8	3	2	3	4	4	3
9	4	3	0	3	4	2
10	4	3	1	4	4	1
11	2	1	4	5	2	2
12	6	5	1	5	4	3
13	4	5	0	3	4	2
14	2	4	2	4	4	3
15	7	1	1	2	4	2
$\bar{x}$	3.8	3.1	2.1	3.5	3.7	1.7
s	1.7	1.6	1.3	0.9	1.3	1.2
RSD%	46	50	61	26	34	67

5.3 Evaluation of homogeneity and batch characterisation study

5.3.1 Outliers

No suspicious data were detected for the homogeneity study based on the interval of observed cfu values which corresponds well to the data interval obtained by the producer BTF (data not shown). Moreover no values of 0 cfu were obtained. Therefore the complete set of data from the homogeneity study was used.

For the batch characterisation study on XLD agar, two suspicious data sets were detected. Data from Lab 3 and Lab 6 included several cfu values of 0, showed a low mean cfu and a RSD% equalling twice the RSD% as obtained by BTF. These laboratories used the same agar composition and supplier as Lab 2 and 4 and should therefore have obtained similar data. Moreover 0 cfu values are considered to be technically unlikely since production of the material spheres is performed with a flow cytometer. Therefore, we excluded these data sets on a technical basis. Data sets from other laboratories were used as a whole.

5.3.2 Statistical distribution

To analyse the data based on an appropriate statistical distribution three general limitations have to be considered:

1) The **underlying true distribution** is unknown. The normal distribution is not applicable since it is used for continuous data or as the extreme limit of the Poisson distribution in case of large means. The Poisson distribution itself is used for discrete data and is often used as a model for the number of events. However the prerequisite for applying the Poisson distribution is that the number of events is very large and the probability of event occurrence is very small such that their product approaches a constant value [11]. For the CRM discussed here this requirement is not met.
If the statistical distribution is known, main questions are related to the composition of the sample and the probability of the occurrence of a certain composition, given the batch composition and the sample size. In case of an unknown statistical distribution, one asks how the batch is composed, given the sample size and its composition.
2) **No repeatability estimate** is available due to the impossibility of doing replicate analyses on the same vial.
3) **ANOVA** is not well-developed for statistical distributions other than the normal distribution and Poisson distribution, and it is fuzzy due to the (comparably) large variances obtained for a small number of vials. Therefore, following the procedures as described in ISO Guide 35 (which use ANOVA for deriving the major uncertainty contributions such as repeatability, laboratory and method bias) is not reasonable.

As a consequence of the above mentioned limitations, models are needed to find an appropriate way to analyse the data. The most obvious is to consider sampling distributions for random discrete variables. These assume a reservoir of objects with a property which can take only a limited number of values (at least 2). The hypergeometric distribution was proposed [11]. Using this model the procedure is considered error-free. All influences of the procedure are transferred to the batch. The batch has *M* objects, all having the property of containing *i* cfu. The number of cfu can take several values (e.g. 3, 4, 5 and 6). One takes samples of *m* units at random from the batch without replacement and expects to obtain the hypergeometric distribution. One alters the fraction of units containing 3, 4, 5, etc cfu in the batch to obtain the best fit to the experimental distribution.

5.3.3 Implementation of the hypergeometric distribution

Tested vials from the homogeneity tests and the batch characterisation all contain a cfu number from 0 to 8. Each different cfu number represents a different object class *i*. Therefore *i* is set at i = 0 to i = 8. No other object classes were assumed for the batch than those observed for the vials of homogeneity and batch characterisation tests.

The **number of vials with *i* cfu** are given in Table 4. The **experimental probability (p_i)** of frequency of vials in a certain object class *i* was calculated based on the number of tested vials. This value was multiplied by the batch size to obtain the **most probable numbers (m.p.n.)** of vials per object class. The probability (P) of this sample pattern or distribution is (relatively) small due to the considerable number of combinations from several classes. The **P** was **maximised** by altering the fractions of the objects in the different classes in the batch (using the MS-Excel® solver function). This resulted in the **final numbers** for the batch.

Taking into account the proportion of the sample size (*m*) to the batch size (*M*), one obtains an **estimate** and a **variance for the number fraction** f_i of each object class *i* in the batch.

The **mean cfu estimate** for the batch ($\bar{i}$) is then calculated according to equation (1) where *i* is the number of cfu in an object class and f_i is the number fraction of this object class in the batch.

$$\bar{i} = \frac{\sum_i i \cdot f_i}{m} \quad (1)$$

The *u* is calculated by equation (2) where *u* (f_i) is the square root of the variance estimate for f_i obtained from the hypergeometric distribution.

$$u(\bar{i}) = \sqrt{\sum_i i^2 \cdot u^2(f_i)} \quad (2)$$

Calculations using the hypergeometric distribution can be found in Table 4A (homogeneity) and Table 4B (batch characterisation). Results are given separately for colony counting on NA and colony counting on XLD agar. For analysis of batch characterisation data, the batch size was decreased by 120 units taking into account the removal of 60 units each for the sample size of homogeneity and stability studies. Batch characterisation data sets of different laboratories were in the same data interval and were obtained with the same procedure. Since the procedure is considered error-free in the hypergeometric distribution model, these data sets were pooled thereby increasing the sample size for statistical analysis.

Table 4. Statistical analysis of homogeneity and batch characterisation data

A. Homogeneity

S. enteritidis on NA

batch size 800
sample size 30

i	0	1	2	3	4	5	6	7	8	9	10	total
number of vials with i cfu	0	0	1	3	4	15	4	2	1	0	0	30
experimental p_i	0	0.00	0.03	0.10	0.13	0.50	0.13	0.07	0.03	0	0	
m.p.n. in class i	0	0.0	26.7	80	107	400	107	53.3	26.7	0	0	800
P of this distribution	0.000496											
final numbers			26	80	107	401	107	53	26			800
number fraction f_i	0	0	0.98	3	4.01	15	4.01	1.99	0.98	0	0	
variance for f_i	0	0	0.95	1.61	1.83	2.69	1.83	1.34	0.95	0	0	
mean cfu ($\bar{i}_{hom}$)	**4.93**											
u_{hom}	0.77											

S. enteritidis on XLD agar

batch size 800
sample size 30

i	0	1	2	3	4	5	6	7	8	9	10	total
number of vials with i cfu	0	4	6	6	7	3	4	0	0	0	0	30
experimental p_i	0	0.13	0.20	0.20	0.23	0.10	0.13	0	0	0	0	
m.p.n. in class i	0	107	160	160	187	80	107	0	0	0	0	800
P of this distribution	0.000498											
final numbers		107	160	160	186	80	107					800
number fraction f_i	0	4.01	6	6	6.98	3	4.01	0	0	0	0	
variance for f_i		1.83	2.15	2.15	2.27	1.61	1.83	0	0	0	0	
mean cfu ($\bar{i}_{hom}$)	**3.37**											
u_{hom}	0.61											

B. Batch characterisation

S. enteritidis on NA

batch size 680
sample size 90

i	0	1	2	3	4	5	6	7	8	9	10	total
number of vials with i cfu	1	0	8	12	21	29	14	3	2	0	0	89
experimental p_i	0.01	0	0.09	0.13	0.23	0.32	0.16	0.03	0.02	0	0	
m.p.n. in class i	7.56	0	60.4	90.7	159	219	106	22.7	15.1	0	0	680
P of this distribution	0.00000938											
final numbers	8	0	60	90	159	220	106	22	15	0	0	680
number fraction f_i	1.06	0	7.94	11.9	21	29.1	14	2.91	1.99	0	0	
variance for f_i	0.95	0	2.51	3	3.74	4.14	3.21	1.56	1.3	0	0	
mean cfu ($\bar{i}_{char}$)	**4.46**											
u_{char}	0.41											

S. enteritidis on XLD agar

batch size 680
sample size 60

i	0	1	2	3	4	5	6	7	8	9	10	total
number of vials with i cfu	0	5	9	12	22	8	2	2	0	0	0	60
experimental p_i	0	0.08	0.15	0.20	0.37	0.13	0.03	0.03	0	0	0	
m.p.n. in class i	0	56.7	102	136	249	90.7	22.7	22.7	0	0	0	680
P of this distribution	0.0000572											
final numbers		57	101	135	252	90	23	22	0	0	0	680
number fraction f_i	0	5.03	8.91	11.9	22.2	7.94	2.03	1.94	0	0	0	
variance for f_i	0	2.05	2.63	2.95	3.57	2.51	1.34	1.31	0	0	0	
mean cfu ($\bar{i}_{char}$)	**3.55**											
u_{char}	0.42											

The same procedure is used for homogeneity testing (IRMM) and batch characterisation (IRMM and external labs). Consequently homogeneity test and batch characterisation results can be treated on an equal level, i.e. the final estimate of the certified value is the simple mean of the $\bar{i}$ values for homogeneity ($\bar{i}_{hom}$) and batch characterisation ($\bar{i}_{char}$). Final cfu values for colony counting on NA as well as XLD agar are given in Table 5. Both homogeneity test and batch characterisation results were different for the NA and the XLD agar. Therefore a quite considerable performance difference of both agars was concluded.

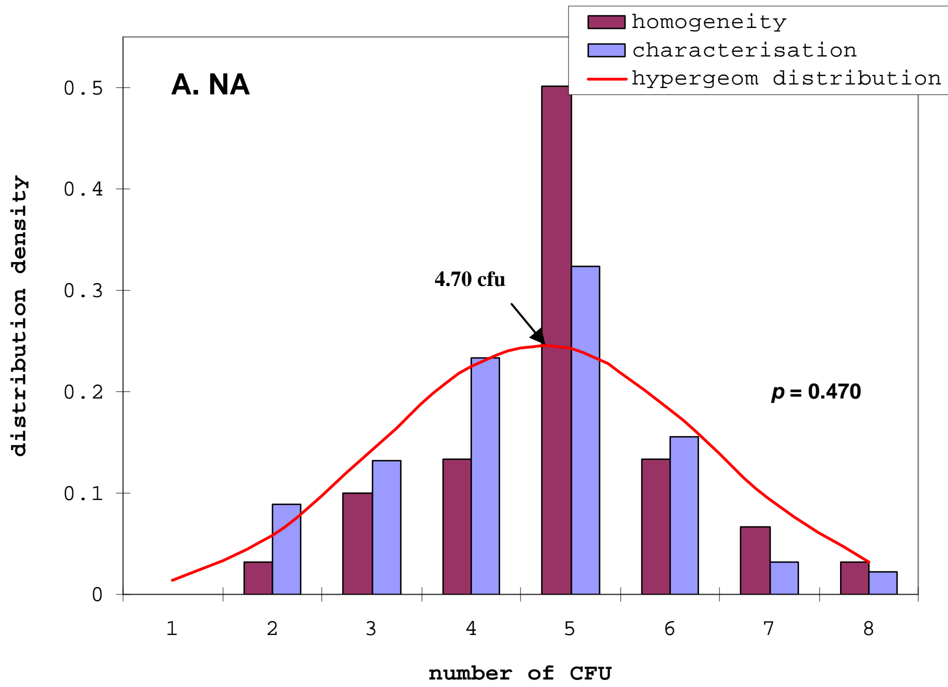
Table 5. **Overview of mean cfu values for homogeneity and batch characterisation.**

	NA	XLD
Homogeneity ($\bar{i}_{hom}$)	4.93	3.37
Characteristation ($\bar{i}_{char}$)	4.46	3.55
Mean	**4.70**	**3.46**

The above conclusions are supported by a simplified model comparing the expected and the observed probabilities. If one takes a model which assumes a hypergeometric distribution with only two possible events (cfu present or absent), and each sample taken comprises 10 objects (m = 10), then the full number of possible events for a batch size of 800 vials is therefore 800 samples * 10 objects = 8000.

In Fig. 1A, representing homogeneity and batch characterisation results on NA, the observed probabilities for finding 1, 2, 3, … cfu in a sample are displayed separately by columns. Given the observed distribution, one may look for parameters of the hypergeometric distribution which match it best. This is normally done in a fit procedure which minimises a certain criterion. Chi square according to the Kolmogorov criterion is suitable for comparing experimental and theoretical distributions, in particular because of it is distribution-independent. 8 test classes were considered for the Kolmogorov test. Object class 0 was excluded since these value was observed only once. Including it as test class would considerably reduce the power of the Kolmogorov test.

Minimisation of the statistic of the chi square goodness-of-fit test was done using the MS-Excel® solver function by altering the p value i.e. the experimental probability of picking an object possessing the specified property. The corresponding hypergeometric distribution was plotted and the optimum p value associated with the distribution is used for further calculations. Indeed the mean of a hypergeometric distribution is p * m with m being the sample size (10 objects). The mean for the batch, 4.70 cfu, agrees well with the values as determined above. Likewise, the observed probabilities and hypergeometric distribution for homogeneity and batch characterisation results on XLD agar are displayed in Fig. 1B. Again the mean for the batch, 3.46 cfu, agrees well with the value in Table 5.



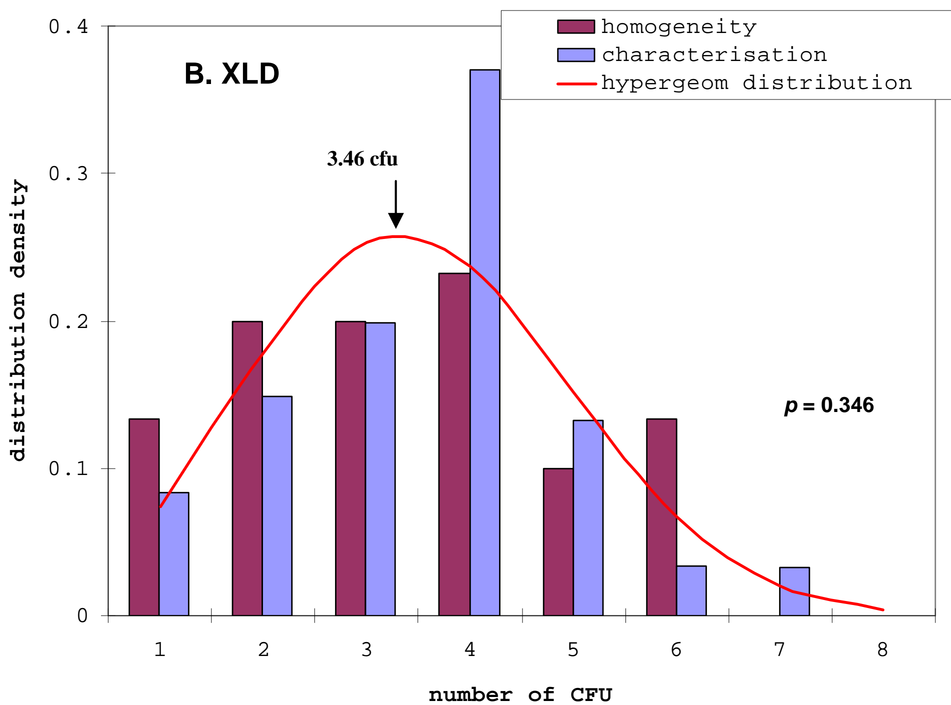


Fig. 1: Representation of observed (histograms) and expected (hypergeometric distribution) cfu values obtained by colony counting on NA and XLD agar. Mean cfu values are indicated by arrows

6 *Stability*

6.1 Planning and results of stability study

The proper conditions for transport of the material to the customer were determined from a short term stability (STS) study of vials stored at 4 °C and -20 °C with time points of 0, 1 and 2 weeks. The stability of the material during storage was determined from long term stability (LTS) studies of vials stored at 4 °C and -20 °C with time points of 0, 3 and 6 months. For each temperature/time combination four vials were used. The short term and long term stability studies were not carried out in an isochronous way. At the end of an incubation period, vials were tested immediately and were not brought back to the storage temperature of -70 °C since this shift could possibly have a negative effect on the viability of the bacteria. Cfu values per material sphere were determined by colony counting on NA plates after incubation at 37 °C for 15–22 h. In parallel with the long term stability study, a postcertification (PC) study was started to allow for further monitoring of the stability of the batch after certification. Vials were tested at 4 °C and -20 °C at time points of 0, 12, 18 and 24 months. For each temperature/time combination three vials were used.

To investigate whether stressing bacteria by storing them at -20 °C results in more difficult growth on a selective agar, a long term stability study was carried out at -20 °C with time points of 0, 3 and 6 months. As for long term stability on NA, vials were tested immediately after each incubation period. For each temperature/time combination four vials were plated on XLD agar. The cfu values per material sphere were determined by colony counting.

Results of stability studies can be found in Tables 6, 7 and 8.

Table 6. STS data of IRMM-352 stored at two different temperatures. At each temperature/time combination four vials were tested on NA. Samples for STS were taken at random from the batch. t = time in weeks.

	cfu per material sphere		
temperature (°C)	**t=0**	**t=1**	**t=2**
4	5	7	3
4	4	2	5
4	2	4	4
4	5	2	3
$\bar{x}$	**4**	**3.8**	**3.8**
-20	5	5	4
-20	4	4	2
-20	2	5	6
-20	5	4	1
$\bar{x}$	**4**	**4.5**	**3.3**

Table 7. LTS data of IRMM-352 stored at two different temperatures. At each temperature/time combination four vials were tested. Samples for LTS were taken at random from the batch. t = time in months.

A. NA

	cfu per material sphere		
temperature (°C)	**t=0**	**t=3**	**t=6**
4	3	0	0
4	3	0	0
4	4	0	1
4	4	2	6
$\bar{x}$	**3.5**	**0.5**	**1.8**
-20	3	2	4
-20	3	2	4
-20	4	5	6
-20	4	5	3
$\bar{x}$	**3.5**	**3.5**	**4.3**

B. XLD

	cfu per material sphere		
temperature (°C)	**t=0**	**t=3**	**t=6**
-20	5	6	2
-20	5	2	5
-20	1	3	3
-20	5	4	3
$\bar{x}$	**4**	**3.8**	**3.3**

Table 8. Available PC data of IRMM-352 stored at two different temperatures. At each temperature/time combination three vials were tested. Samples for PC were taken at random from the batch. t = time in months.

	cfu per material sphere		
temperature (°C)	**t=0**	**t=12**	**t=18**
4	5	0	2
4	4	0	2
4	3	1	0
$\bar{x}$	**4**	**0.3**	**1.3**
-20	5	4	3
-20	4	3	3
-20	3	3	2
$\bar{x}$	**4**	**3.3**	**2.3**

6.2 Evaluation of stability data

For assessing stability in this study, one would normally look at the experimental statistical distributions obtained at several times and temperatures and decide at which point in time these distributions start differing significantly from the hypergeometric distribution obtained for the batch. Unfortunately, the low number of vials at each sampling point results in a large heterogeneity and variance among the data sets and therefore in large uncertainties. This

low number of data points is due to small batch size (800 vials) and the absence of replicate analysis.

Because of the limitations as explained above, data were not plotted but conclusions were drawn from the raw data. At 4 °C the material is not stable since three cfu values of 0 were found already after 3 months. Therefore the material should be shipped frozen. At -20 °C cfu values are close to the certified value at any point in time, on NA as well as XLD agar, and the material can be stored at -20 °C for at least 6 months. Available data on post-certification monitoring at 4 °C and -20 °C, shown in Table 8, support the stability of the material at -20°C for even longer periods of time. At time points of 12 months as well as 18 months, three vials for each temperature were analysed. At 18 months, data for -20 °C are still in the normal range and no cfu values of 0 are obtained. However, an uncertainty estimate has to be included in the final *U* budget. Because of the lack of data at 4 °C and -20 °C, this can only be done based on additional data at storage temperature (-70 °C).

6.3 Stability at storage temperature

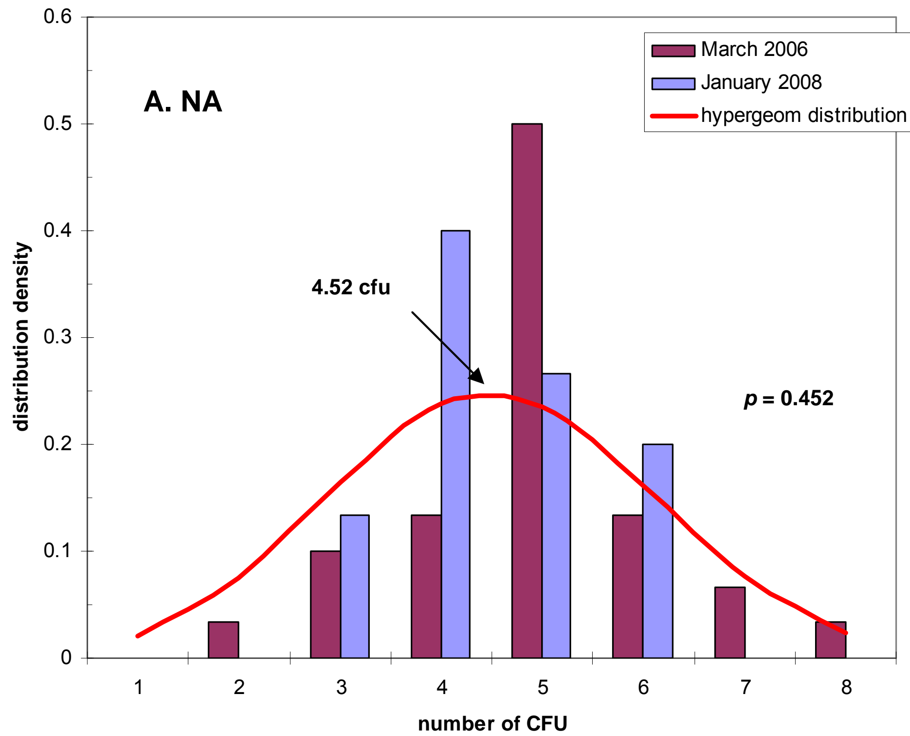
No long term stability study was carried out at the storage temperature (-70 °C) and no intermediate sampling points are available. However, after the certification campaign 2 times 15 vials were taken at random from the batch stored at -70 °C and analysed on NA and XLD agar. The study was non-isochronous but refers to the initial state as established by the homogeneity test in March 2006 and the study end point in either November 2007 (NA) or January 2008 (XLD).

Results of the study are given in Table 9 and are presented graphically in Fig. 2 using histograms according to the model as described in section 5.3.3. Hypergeometric distributions for the start and end point were fitted to the histograms using the minimum criterion for the chi square value(s). The distributions are given in the figures for the data obtained at the end point of the stability study. It can clearly be seen that changes in the distributions occur even at that low storage temperature. The question whether the distribution at the end point significantly deviates from the one at the beginning can best be answered by looking at the chi square value obtained for a comparison of the end-point histogram and the best-fit hypergeometric distribution at the beginning. If this value exceeds the critical value (degrees of freedom at the end point are number of test classes – 2, i.e. 6, which gives a critical value of 12.6), the observed instability is significant. The corresponding chi square values are 5.03 for NA and 7.89 for XLD. This means that the observed changes are not significant.

The mean cfu values for the hypergeometric distribution at the end point are calculated from the optimum *p* value ($p * m$) and are 4.52 cfu on NA and 3.86 cfu on XLD.

Table 9. Measurement data for stability monitoring of IRMM-352 at storage temperature (-70 °C). Individual data per vial as well as the mean ($\bar{x}$), standard deviation (*s*) and relative standard deviation (RSD%) are shown.

NA		XLD	
Vial identification number	**Measured value (cfu)**	**Vial identification number**	**Measured value (cfu)**
101	6	140	4
133	3	166	3
160	6	191	4
198	4	209	5
213	5	238	5
241	4	254	1
250	5	288	5
285	4	330	5
327	4	354	3
343	5	374	3
372	4	408	4
400	5	434	5
455	4	461	3
472	6	731	3
751	3	741	4
$\bar{x}$	4.5	$\bar{x}$	3.8
s	1.0	***s***	1.2
RSD%	22	**RSD%**	30



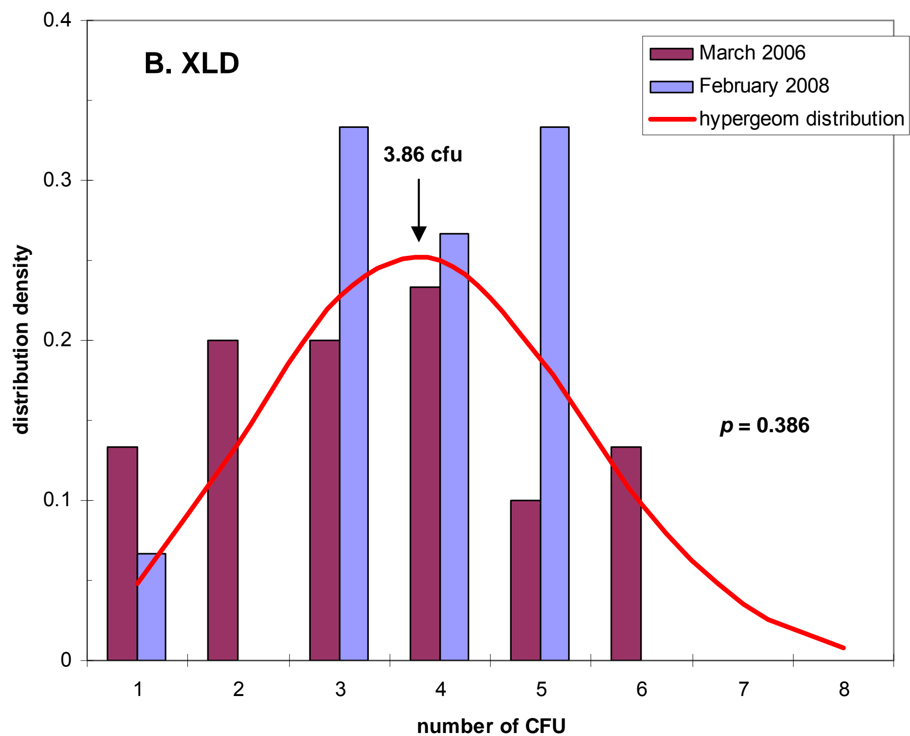


Fig. 2: Representation of observed cfu values (histograms) for start and end point data at -70 °C. The hypergeometric distribution (expected values) is plotted for the end point data. Mean cfu values at the end point are indicated by arrows.

To include an estimate in the final uncertainty budget and to apply a shelf-life, an average degradation rate c_{dg} was calculated using the mean cfu for the batch (i_{cert}) and the mean cfu at the end of the stability study at -70 °C (i_{end}). Assuming that the degradation process is governed by an exponential law, c_{dg} is obtained as

$$c_{dg} = \frac{\ln(i_{cert}) - \ln(i_{end})}{\Delta t_{study}}$$

where Δt_{study} denotes the duration of the study (22 months on NA and 23 months on XLD). Assuming continuation of this process, the mean cfu content i_{est} at the end of the expected shelf-life t_{shelf} (54 months) was calculated according to

$$\ln(i_{est}) = \ln(i_{cert}) - c_{dg} \cdot t_{shelf}$$

Half of the difference $\Delta_{0.5}$ between i_{cert} and i_{est} fully covers the observed difference. On the other hand, both i_{cert} and i_{est} have an uncertainty which should be estimated as being not smaller than the precision observed in the homogeneity study s_{hom} which had been carried out under repeatability conditions. Therefore u_{lts} was calculated as follows (Table 10):

$$u_{lts} = s_{\text{hom}} / \sqrt{2} \quad if \quad \Delta_{0.5} \le s_{\text{hom}}$$
$$u_{lts} = \sqrt{\Delta_{0.5}^2 - s_{\text{hom}}^2} \quad if \quad \Delta_{0.5} > s_{\text{hom}}$$

Table 10: Values for i_{cert}, i_{end}, c_{dg}, i_{est}, and u_{lts} for stability monitoring at -70 °C on NA and XLD

	i_{cert}	i_{end}	c_{dg}	i_{est}	u_{lts}
NA	4.70	4.52	0.0018	4.27	0.54
XLD	3.46	3.86	-0.0048	4.48	0.43

7 *Additional characterisation*

The DNA sequence of *sef*A is characteristic for *S. enteritidis* and can be used for detection, identification and discrimination from other *Salmonella* strains [8]. Three random vials from the batch were analysed by amplification of the *sef*A gene by PCR using primers SEFA2 and SEFA4. The PCR product was purified and cloned into pCR2.1. Purified plasmid DNA containing the 331 bp amplicon of the *sef*A gene was purified and used for DTCS using sequencing primer M13-Forward (Invitrogen, Merelbeke, BE). The Basic Local Alignment Search Tool (BLAST) finds regions of local similarity between sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches. Using nucleotide-nucleotide BLAST the obtained sequence was compared to other nucleotide sequences in the GenBank database and showed 100 % sequence identity with the reported DNA sequence of the *sef*A gene (Genebank Accession no. X98516) (Fig. 3).

```
gi|1405814|emb|X98516.1|SESEFAG  S.enterica sefA gene
Length=1416

 Score =  656 bits (331),  Expect = 0.0
 Identities = 331/331 (100%), Gaps = 0/331 (0%)
 Strand=Plus/Minus

Query  96    GGGACATTTAGCGTTTCTTGAGAGCTGGCAACTCGCCAACCTGCAAGCCCGTCAATTCCA  155
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1337  GGGACATTTAGCGTTTCTTGAGAGCTGGCAACTCGCCAACCTGCAAGCCCGTCAATTCCA  1278

Query  156   GTATTTGCTTGGTCATTAATATTGGCTCCCTGAATACGCCCACGGAAAACAGGCTGTCCT  215
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1277  GTATTTGCTTGGTCATTAATATTGGCTCCCTGAATACGCCCACGGAAAACAGGCTGTCCT  1218

Query  216   TGTCCATCAACGAACGGGACAGTGGCTACACCACCAGATACCGAAGCCCCTTTACCTGCA  275
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1217  TGTCCATCAACGAACGGGACAGTGGCTACACCACCAGATACCGAAGCCCCTTTACCTGCA  1158

Query  276   ATAGATACTGAGTTATGTGGACCAGTAGCAGTAATGCTGAGAGTACCAACTTTCTGACCA  335
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1157  ATAGATACTGAGTTATGTGGACCAGTAGCAGTAATGCTGAGAGTACCAACTTTCTGACCA  1098

Query  336   GCAGCAACAGCAGGCCCTGTAAAGCCAGGATCCTGACTCCAGTTGGCTGATGTTGTATTC  395
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1097  GCAGCAACAGCAGGCCCTGTAAAGCCAGGATCCTGACTCCAGTTGGCTGATGTTGTATTC  1038

Query  396   TGAGCTGCAATAGTAACCGCTGCCTGAACCT  426
             |||||||||||||||||||||||||||||||
Sbjct  1037  TGAGCTGCAATAGTAACCGCTGCCTGAACCT  1007
```

Fig. 3 Comparison of the DNA sequence of the *sef*A amplicon obtained from IRMM-352 (Query) with the reported DNA sequence of the *fli*C gene (Genbank accession no. X98516) using Nucleotide-nucleotide BLAST (blastn) on www.ncbi.nlm.nih.gov.

8 Certified values and uncertainties

8.1 Certified values and uncertainty budget

Since data are not normally distributed, common ANOVA strategies do not apply. The certified number of cfu per material sphere of IRMM-352 was calculated as the mean of the mean cfu values in the homogeneity study and batch characterisation. These mean values were calculated based on a hypergeometric distribution. The expanded uncertainty (*U*) of the certified value is calculated from the contributing standard uncertainties from characterisation, homogeneity test and stability studies using equation (3) and a coverage factor *k* = 2. The uncertainty corresponds to a level of confidence of about 95 %.

$$U_{CRM} = 2.\sqrt{u_{\mathrm{hom}}^2 + u_{char}^2 + u_{lts}^2} \quad (3)$$

Table 11 gives an overview of (rounded) certified values, standard uncertainties and expanded uncertainties for colony counting on NA as well as XLD. Given the general limitation that the measurand can have only integer values, the certified value and uncertainty are rounded (upwards) to the closest integer.

Table 11: Certified values, standard uncertainties (*u*) and expanded uncertainty (*U*) for IRMM-352 on NA and XLD

NA	mean	*u*
homogeneity	4.93	0.77
characteristation	4.46	0.41
stability		0.54
total, *U* at the 95% level	4.70	2.05
total (rounded, 95%)	**5**	**2**
XLD agar	mean	*u*
homogeneity	3.37	0.61
characteristation	3.55	0.42
stability		0.43
total, *U* at the 95% level	3.46	1.70
total (rounded, 95%)	**4**	**2**

8.2 Interpretation and use of the certified values

If this CRM is used for presence/absence tests, the absolute minimum of samples to be analysed should be two. Conclusions should be based on individual cfu values. If the result is within the 95% confidence interval specified for the CRM, then the test has been passed, if not, it failed.

If this CRM is used for method validation or testing of media, a similar approach as for certification of the batch should be applied. This requires the measurement of an appropriate number of CRM vials, minimum 15 in agreement with the number of CRM vials analysed during the characterisation study (section 5.2). Conclusions should be based upon patterns (histograms) of the results obtained in the laboratory and during certification rather than on mean cfu values. The histogram obtained in the laboratory is compared with the hypergeometric distribution obtained for the homogeneity and batch characterisation data (section 5.3.3) and a chi square value is calculated. The success of the validation is assessed from this chi square value with respect to critical limits. If the lab falls short of the critical value, it failed in method validation.

9 Metrological traceability

The certified value (cfu per material sphere) is traceable to the SI unit 1 applying the procedures ISO 7218 and ISO 6579. ISO 7218 [5] provides general rules for microbiological examinations and ISO 6579 [6] describes a horizontal method for the detection of *Salmonella* spp. The sequence identity of the gDNA has been confirmed by DTCS of the *sef*A gene [8].

10 Instructions for use

10.1 Dispatch

Dispatch to the customer must be done at -20 °C. Upon receipt by the customer, the material should be used immediately or can be stored at -20 °C or -70 °C. No instability of the material was detected when stored at -20 °C for up to 12 months and at -70 °C for up to 54 months.

10.2 Instructions for use

Bacterial samples do not survive at room temperature. Therefore take vials out of the freezer one by one during analysis and handle vials with care. Use sterile consumables in all steps and work under sterile conditions.

1. Dry one appropriate media plate per material sphere.
2. To open the vial containing a material sphere, aseptically remove stopper.
3. Tip the material sphere into the centre of the plate. Inspect the empty vial and make sure no remaining parts of the material sphere are left.
4. Rehydrate by pipetting 100 µL of 0.9% (m/v) NaCl directly onto the material sphere. Wait 30 seconds for the material to dissolve.
5. Use one sterile disposable spreader (no glass spreaders) to evenly spread the dissolved material carefully over the plate surface, avoiding the borders of the plate.
6. Move the end of the spreader lightly up and down on the agar surface 5 times to help remove any excess fluid on the spreader.
7. Dry plates for 30 minutes at room temperature before inverting.
8. Incubate at appropriate time and temperature.

11 References

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Acknowledgements

The authors would like to thank D. Charels and A. Bernreuther (IRMM) for reviewing of the certification report, as well as the experts of the Certification Advisory Panel 'Biological Macromolecules and Biological/Biochemical Parameters', R. Dybkaer (Frederiksberg Hospital, DK), E. Jansen (National Institute for Public Health and Environment, NL) and U. Örnemark (LGC Standards, SE) for their critical comments.

Annex 1: Microbiological CRMs available from IRMM

BCR-506: Capsules filled with milk powder artificially contaminated by *Enterococcus faecium* (WR 63 / NCTC 13169)

BCR-507R: Capsules filled with milk powder artificially contaminated by *Salmonella typhimurium* (ALM 40 / NCTC 13171)
Colony forming particles per capsule / number of negative capsules

BCR-527: Capsules filled with milk powder artificially contaminated by *Enterobacter cloacae* (WR 3 / NCTC 13168)

BCR-528: Capsules filled with milk powder artificially contaminated by *Bacillus cereus* (ATCL 9139)

BCR-594: Capsules filled with milk powder artificially contaminated by Escherichia coli (WR 1 / NCTC 13167)

BCR-595: Capsules filled with milk powder artificially contaminated by Listeria monocytogenes (Scott A) (ALM 92 / NCTC 13173)

European Commission

EUR 23541 EN – Joint Research Centre – Institute for Reference Materials and Measurements
Title: Certification of a reference material with *Salmonella enteritidis* (NCTC 12694) at level of 5 colony forming units on nutrient agar and 4 colony forming units on xylose lysine deoxycholate agar, IRMM-352
Author(s): L. De Baets, P. van Iwaarden, W. Bremser, N. Meeus, W. Philipp, H. Schimmel
Luxembourg: Office for Official Publications of the European Communities
2008 – 35 pp. – 21.0 x 29.7 cm
EUR – Scientific and Technical Research series – ISSN 1018-5593
ISBN 978-92-79-10272-1
DOI 10.2787/85196

Abstract

This report describes the certification of a reference material (IRMM-352) of *Salmonella enteritidis*. Certified Reference Materials (CRMs) for microbiological analysis are indispensable tools for development and validation of detection methods and for the implementation and support of internal and external quality control in the area of microbiological analysis. Each vial contains one material sphere of *S. enteritidis*. The homogeneity and stability (at 4 °C, -20 °C and -70 °C) of the batch was assessed by monitoring colony forming units (cfu) on nutrient agar (NA) and xylose lysine deoxycholate (XLD) agar of selected vials by colony counting. The material is not stable at 4 °C but no instability was detected when stored at -20 °C for up to 12 months and at -70 °C for up to 54 months. The batch was characterised by six laboratories to determine a certified value of cfu per vial on NA and XLD agar. A quite considerable performance difference of both agars was observed. The certified value is 5 cfu on NA with an expanded uncertainty of 2 and 4 cfu on XLD agar with an expanded uncertainty of 2 using a coverage factor $k = 2$, corresponding to a level of confidence of about 95 %. DNA sequence analysis of the coding region for the *sef*A gene identified the material as *S. enteritidis*.

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