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Certification of a reference material with *Enterococcus faecalis* (CIP 106877) at a target level of 1000 colony forming units per material sphere, IRMM-355

L. de Baets, N. Meeus, H. Schimmel

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IRMM information
REFERENCE MATERIALS

Certification of a reference material with *Enterococcus faecalis* (CIP 106877) at a target level of 1000 colony forming units

IRMM-355

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Abstract

This report describes the certification of a reference material (IRMM-355) of *Enterococcus faecalis*. 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. The content of each vial of IRMM-355 is one material sphere containing *Enterococcus faecalis*. The homogeneity and stability (at -20 °C and -70 °C) of the batch was assessed by counting colony forming units (cfu) per material sphere on horse blood agar (BA) and Slanetz and Bartley agar (SB agar). The material is stable when stored at a maximum temperature of -20 °C for up to 18 months. The batch was characterised by six laboratories to establish a certified value of cfu per material sphere on BA and SB agar. The certified value for BA is 890 cfu with an expanded uncertainty of 135. The certified value for SB agar is 823 cfu with an expanded uncertainty of 126. In both cases, a coverage factor *k* = 2 is used, corresponding to a level of confidence of about 95 %. The identity of the bacteria was confirmed by DNA sequence analysis of the coding region for the Enterococcus faecalis endocarditis antigen (*efa*A).

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Glossary

BA	(horse) blood agar
BLAST	Basic Local Alignment Search Tool
b	slope of the regression line
bp	base pair
cfu	colony forming unit
cfu_{sphere}	colony forming units per material sphere
CRM	Certified Reference Material
dNTP	deoxynucleotide triphosphate
DNA	deoxyribonucleic acid
*efa*A	Enterococcus faecalis endocarditis antigen
gDNA	genomic DNA
i	portion of 100 µL
IRMM	Institute for Reference Materials and Measurements
j	portion of 50 µL
k	coverage factor
LTS	long-term stability
N	total number of vials in the homogeneity study
n	number of laboratories
NCTC	national collection of type cultures
PCR	polymerase chain reaction
RM	reference material
RSD	relative standard deviation
s	standard deviation
SB agar	Slanetz and Bartley agar
s_{bb}	standard deviation between bottles
u_b	standard uncertainty of the slope of the regression line
u_{bb}	standard uncertainty of between-bottle homogeneity
u_{char}	standard uncertainty related to the characterisation
u_{lts}	standard uncertainty related to the long-term stability of the material
u_c	combined standard uncertainty
U_{CRM}	expanded uncertainty of the CRM
$\bar{x}$	mean
$\bar{y}$	mean of the homogeneity study

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 already offers microbiological reference materials (RMs) with certified colony forming unit (cfu) numbers[1] per capsule covering six different food and water micro-organisms (http://irmm.jrc.ec.europa.eu). Measurement effects, such as Poisson distribution at low cfu counts, encouraged us to consider alternative approaches for development and certification of reference materials. The production of freeze-dried BioBalls™ is not based on aliquoting but rather on transferring a counted number of bacterial or yeast cells into a single BioBall™ with a modified flow cytometer [1]. The IRMM's portfolio of CRMs in the BioBall™ format currently consists of two low cfu materials (IRMM-351, containing *E. coli* O157 and IRMM-352, containing *S. enteritidis*) and one material at a target level of 1000 cfu (IRMM-354, containing *C. albicans*). IRMM currently extends the range of these CRMs to more micro-organisms at a level of 1000 cfu per material sphere for water control laboratories. *Enterococcus faecalis* is a gram-positive, opportunistic bacterium that inhabits the gastrointestinal tracts of humans and many animals. Because of their ability to acquire high-level resistance to antimicrobial agents, enterococci have emerged as nosocomial pathogens worldwide. *Enterococcus faecalis* causes 80 to 90% of human enterococcal infections [2, 3].

This report describes the certification of a batch of 1000 vials (IRMM-355) containing *E. faecalis* at a target level of 1000 cfu per material sphere. Homogeneity and stability of the produced batch were analysed at IRMM following in house procedures applying a quality management system according to ISO Guide 34 [4-7]. Batch characterisation was performed by IRMM and five external laboratories. The methods used for the batch characterisation as well as for homogeneity and stability testing were colony counting on Horse Blood Agar (BA) and Slanetz and Bartley agar (SB agar) according to ISO 7218 [8] and ISO 7899-2 [9] respectively. BA is Columbia agar containing 5 % horse blood and is used as a non-selective growth agar. SB agar is a selective growth agar for the detection and enumeration of intestinal enterococci in surface and waste water.

Identification of *E. faecalis* was performed by DNA sequence analysis of the *efa*A gene encoding the endocarditis antigen [10].

[1] Numbers refer to entitic numbers throughout the whole report

2 Participants

The reference material (RM) was developed and processed by BTF Pty Ltd. (North Ryde, Australia). Analysis of homogeneity and stability and additional characterisation of IRMM-355 was performed at IRMM. Participants for batch characterisation measurements were:

- IRMM, Joint Research Centre, European Commission, Geel, BE
- CCFRA Technology Ltd, Gloucestershire, GB (ISO/IEC 17025 accreditation)
- Leatherhead Food International, Leatherhead, GB (ISO/IEC 17025 accreditation; UKAS 1152)
- RIKILT, Wageningen, NL (ISO/IEC 17025 accreditation; RvA L-014)
- Silliker Iberica, Barcelona, ES (ISO/IEC 17025 accreditation; ENAC 257/LE413)
- Food and Environment Research Agency, York, GB

3 Processing

IRMM-355 was processed in the BioBall™ format [1]. A modified flow cytometer was used, allowing the sorting and counting of a number of cultured bacterial cells into a single droplet. For the RM discussed here the target value was set at 1000 cells per droplet. In a second step the droplets are collected in a liquid nitrogen container resulting in frozen spheres. Finally each sphere was transferred into a labelled glass vial and 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 under vacuum with a rubber stopper and stored at -70 °C until analysis. In total, 1000 vials (labelled from 200 to 1199) each containing one material sphere were produced. Since droplets are collected together in the same liquid nitrogen container before freeze-drying, labelling in the order of manufacturing is not possible. Hence, numbering of the vials does not correspond to the production sequence.

4 Procedures

4.1 Minimum sample volume

One material sphere is used per measurement according to the instructions of BTF.

4.2 Colony counting

Plating and colony counting was performed on BA according to ISO 7218 [8] and on SB agar according to ISO 7899-2 [9].

1 mL of an aqueous solution of NaCl (mass concentration of 0.9 g/L) was added to a vial containing one material sphere. For plating on BA, the standard plating technique was used. Nine portions *i* of 100 µL and one portion containing the rest of the solution were spread over 10 agar plates. Plates were incubated at 37 °C ± 1 °C for 14 to 24 h. For plating on SB agar, the membrane filtration technique was used. Nineteen portions *j* of 50 µL and one portion containing the rest of the solution were divided over 20 tubes containing 10 mL of an aqueous solution of NaCl (mass concentration of 0.9 g/L). The solution in each tube was filtered with a 0.45 µm filter and tubes were washed twice with 10 mL of an aqueous solution of NaCl (mass concentration of 0.9 g/L). Each filter was placed on a SB agar plate. Plates were incubated at 36 °C ± 2 °C for 40 h to 44 h. After incubation, colonies on individual plates were counted. To obtain the cfu number per material sphere, counts of 10 respectively 20 individual plates were summed up according to equation (1) or (2) respectively.

$$\text{cfu}_{\text{sphere}} = \sum_{i=1}^{10} cfu_i \qquad (1)$$

$$\text{cfu}_{\text{sphere}} = \sum_{j=1}^{20} cfu_j \qquad (2)$$

4.3 PCR

Each reaction mixture for PCR reactions (25 µL total volume) contained 20 mmol/L Tris-HCl (pH 8.4), 50 mmol/L 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, suspended in 50 µL H_2O, was used. The 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 gDNA at 95 °C for 5 min; 30 cycles denaturation at 95 °C for 60 s, annealing at 58 °C for 60 s, extension at 72 °C for 60 s; incubation at 72 °C for 10 min. PCR products were visualised by agarose gel electrophoresis.

Table 1. Primers used for qualitative PCR.

Organism	PCR target, amplicon length	Primer	DNA sequence	Reference
Enterococcus faecalis	*efa*A gene, 688 bp	*efa*A-f *efa*A-r	5'-GCC AAT TGG GAC AGA CCC TC A-3' 5'-CGC CTT CTG TTC CTT CTT TGG C-3'	[10]

4.4 DNA sequencing

PCR products (25 µL), amplified with primers *efa*A-f and *efa*A-r, 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 BigDye® Terminator v1.1 cycle sequencing reaction, according to instructions of the manufacturer (Applied Biosystems).

Samples were analysed on a ABI Prism® 3130 Genetic Analyser (Applied Biosystems) using the POP-7™ polymer and the RapidSeq36 run module. As a control for the sequencing reaction, the pUC18 plasmid was included on each sequencing plate.

5 *Homogeneity and characterisation of the batch*

5.1 Design of homogeneity study

Vials for the homogeneity study were taken from the stock at -70 °C. 1000 vials were produced and the total number of vials to be used in the homogeneity study (*N*) was calculated from $\sqrt[3]{1000} = 10$ [4]. Homogeneity of the batch was measured by determining the cfu values per material sphere using colony counting on BA, according to ISO 7218 [8] and on SB agar, according to ISO 7899-2 [9] (Section 4.2). Since one vial contains only one material sphere which has to be characterised as a whole, replicate analysis per vial is not possible (Fig.1).

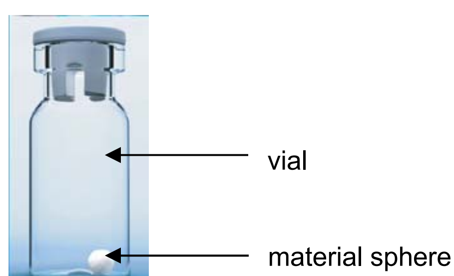


Fig. 1: Representation of a vial of IRMM-355 containing one material sphere. For analysis, a reconstituted material sphere is divided in 10 portions *i* for plating on BA (standard plating) or 20 portions *j* for plating on SB agar (membrane filtration).

5.2 Evaluation of homogeneity study

The results of the homogeneity measurements are summarised in Table 2.

Table 2. Measurement data for the homogeneity study of IRMM-355. Individual data per vial as well as the mean ($\bar{y}$), standard deviation (*s*) and relative standard deviation (RSD) are shown for agars BA and SB agar. Suspicious outliers are marked with *.

BA		SB agar	
Vial identification number	**Measured value (cfu)**	**Vial identification number**	**Measured value (cfu)**
202	880*	**797**	751
282	922	**821**	832
321	838*	**876**	782
388	912	**877**	789
438	895	**938**	817
479	899	**999**	812
539	913	**1040**	736
583	910	**1054**	813
617	914	**1140**	797
663	905	**1179**	829
$\bar{y}$	899	$\bar{y}$	796
s	24	***s***	32
RSD	2.7 %	**RSD**	4.0 %

The mean of the study was $\bar{y}$ = 899 cfu for colony counting on BA and $\bar{y}$ = 796 cfu for colony counting on SB agar. Grubbs tests were performed to detect outlying results. For results on BA, cfu values of 880 and 838 were detected as outliers. However, there is no technical reason for excluding these data. Therefore the complete data set was used. For results on SB agar, no outliers were detected and the complete set of data was used.

Regression analyses were used to evaluate drifts in results related to the analytical sequence. No significant trends were observed in the results. It was furthermore checked whether the data followed a normal and unimodal distribution using normal probability plots and histograms respectively. The individual data were unimodal for both growth agars.

The between bottle standard deviation (s_{bb}) was estimated as follows: as mentioned earlier, no replicate analysis could be applied because the sample intake is a complete material sphere. Therefore the only estimate we have for s_{bb} is the standard deviation of the mean cfu value ($\bar{y}$) i.e. 24 cfu for colony counting on BA and 32 cfu for colony counting on SB agar. The value for s_{bb} was used as an estimate for the uncertainty contribution related to the between-bottle heterogeneity (u_{bb}) (Table 3).

Table 3. Between bottle homogeneity (s_{bb}) for IRMM-355 on BA as well as on SB agar

	Relative between bottle homogeneity ($s_{bb, rel}$)	Absolute between bottle homogeneity (s_{bb}) (cfu)
BA	2.7 %	24
SB agar	4.0 %	32

6 Stability

6.1 Design of stability study

No short term stability study was carried out. It was previously shown for materials IRMM-351, IRMM-352 and IRMM-354 that bacteria in the material spheres are not stable at 4 °C and the materials should be shipped frozen.

The stability of the material during storage was determined from long term stability (LTS) studies of vials stored at -20 °C and at the normal storage temperature of -70 °C. Two LTS studies were run in parallel with time points of 0, 3, 6, 9 months and 0, 12, 18 months respectively. The 18 months study was started to allow for further monitoring of the stability of the batch after certification. For both LTS studies three vials were used for each temperature/time combination. The long term stability studies at -20 °C were not carried out in an isochronous way. The reason for this is the lack of knowledge about the impact of subsequent storage temperatures on the viability and recovery of the bacterial cells. Samples were taken from the stock at -70 °C and stored at -20 °C. At the end of a storage period, material spheres in the vials were tested immediately and were not brought back to the storage temperature of -70 °C.

Cfu values per material sphere were determined by plating and colony counting on BA according to ISO 7218 [8] and on SB agar according to ISO 7899-2 [9]. Plates were incubated at 37 °C ± 1 °C for 14 to 24 h (BA) or at 36 °C ± 2 °C for 40 h to 44 h (SB agar).

6.2 Evaluation of stability data

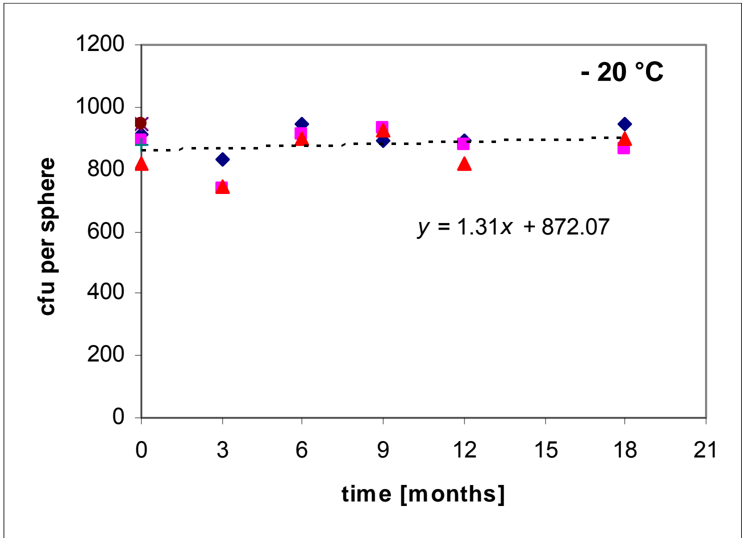
Results of the stability studies are given in Table 4 and plotted in Fig. 2. Since the study set-up was the same for both LTS studies, all 6 measurements at time point 0 were integrated in the same evaluation. Cfu values at a time point of 3 months were detected as outliers for BA at both temperatures and for SB agar at -70 °C. However, cfu values increase again at a time point of 6 months and therefore it was concluded that the decrease at 3 months is not due to instability. Outlying data were therefore retained for calculation of the average cfu value. Since measurements are not performed in an isochronous way, repetition of the measurements is not possible. For plating and colony counting on SB agar, cfu values at a time point of 18 months revealed an increasing trend. Therefore measurements were repeated immediately for 3 additional samples at each temperature and all 6 measurement results are included in the evaluation.

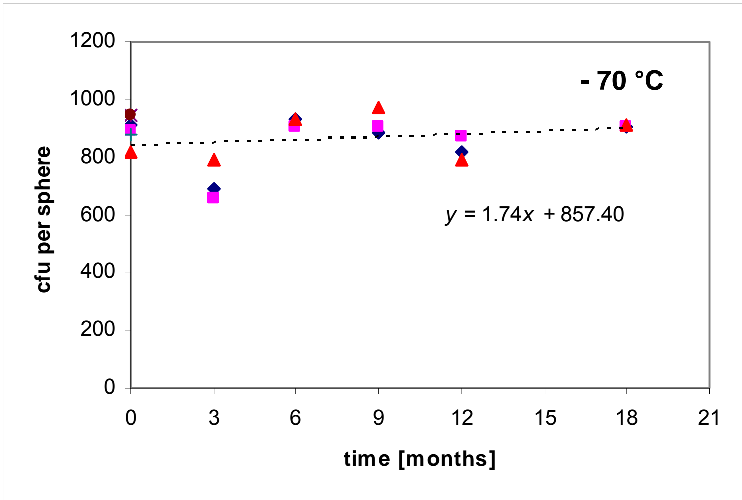
Table 4. LTS data of IRMM-355 stored at two different temperatures. At each temperature/time combination three material spheres were tested. Vials for LTS were taken at random from the batch. *t* = time in months. Suspicious outlying values are marked with *.

BA	Cfu per material sphere					
Temperature [°C]	***t* = 0**	***t* = 3**	***t* = 6**	***t* = 9**	***t* = 12**	***t* = 18**
-20	913	833	946	894	890	943
-20	890	739*	915	932	881	863
-20	821	743*	901	922	816	895
-20	942					
-20	948					
-20	875					
$\bar{x}$	**898**	**772**	**921**	**916**	**862**	**900**
-70	913	690*	933	888	818	903
-70	890	657*	906	905	871	905
-70	821	790	932	969	790	910
-70	942					
-70	948					
-70	875					
$\bar{x}$	**898**	**712**	**924**	**921**	**826**	**906**

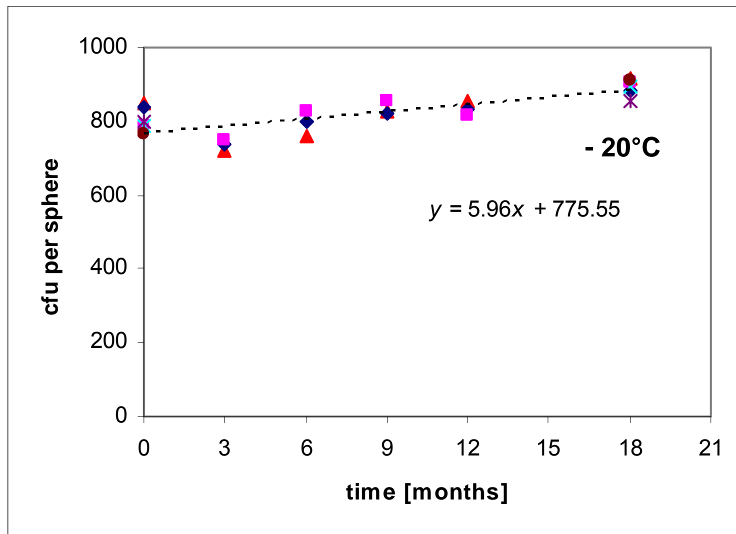
SB agar	Cfu per material sphere					
Temperature [°C]	***t* = 0**	***t* = 3**	***t* = 6**	***t* = 9**	***t* = 12**	***t* = 18**
-20	839	738	797	824	835	883
-20	788	747	827	855	818	903
-20	851	720	762	829	856	914
-20	789					894
-20	798					852
-20	767					908
$\bar{x}$	**805**	**735**	**795**	**836**	**836**	**892**
-70	839	672*	841	836	804	917
-70	788	681*	836	788	858	888
-70	851	715	880	813	813	850
-70	789					924
-70	798					886
-70	767					880
$\bar{x}$	**805**	**689**	**852**	**812**	**825**	**891**

BA





SB agar



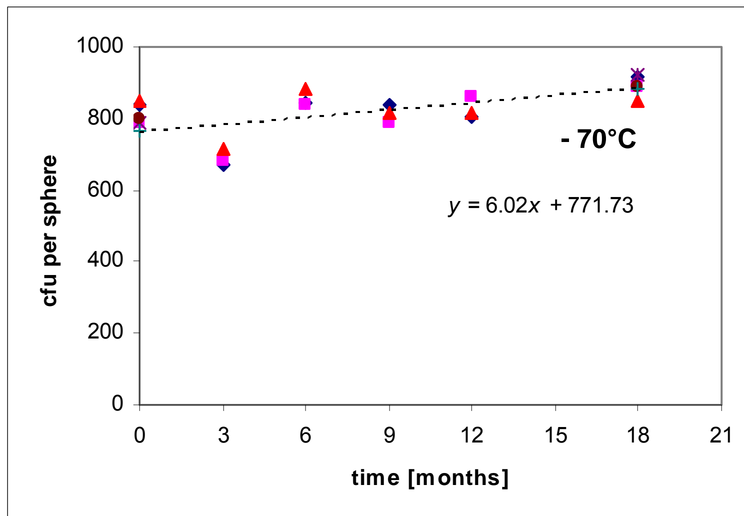


Fig. 2 Graphical representation of LTS data of IRMM-355. At each temperature/time combination three/six material spheres were tested. Different symbols represent the different measurement results at each time point. The dashed lines represent the calculated regression lines from Table 5. Equations for the regression lines are indicated.

6.2.1 Regression analysis

To investigate whether there is a degradation of the material with time, regression lines were calculated and their slopes were tested for significance. For data on BA, the slopes were not significantly different from zero ($|b| / u_b < t_{crit}$) at the investigated level of confidence (Table 5). For data on SB agar, slopes were found to be significantly different from zero at both temperatures. However, the observed trend was not a decreasing one pointing at degradation of the material but mean cfu values at a time point of 18 months were significantly higher than at earlier time points. Bacterial growth at temperatures of -20 °C and -70 °C is impossible. Moreover an increasing trend was not observed for stability data of

released materials IRMM-351, IRMM-352 and IRMM-354. Therefore we attribute the increasing trend to the low number of analysed material spheres and conclude that it is safe to store the material at -20 °C for 18 months.

Table 5. Test for significance of the slope ($|b| / u_b$) after 18 months storage at the specified temperature. Statistically significant data ($t_{crit\ (0.05;\ 16)} > 2.12$) are indicated in bold.

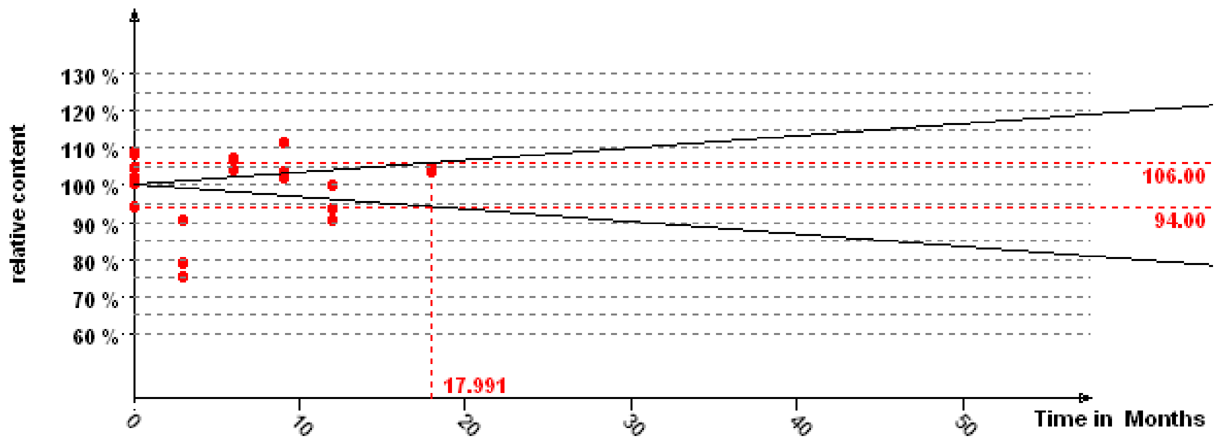
Temperature [°C]	Agar	slope b	intercept a	u_b	$\|b\| / u_b$
-20	BA	1.31	872.07	2.18	0.60
-70	BA	1.74	857.40	2.95	0.59
-20	SB agar	5.96	775.55	1.08	**5.52**
-70	SB agar	6.02	771.73	1.55	**3.88**

6.2.2 Shelf-life and uncertainty

The initially predicted shelf-life (storage time) of IRMM-355, for which the certified values are valid, was calculated from the available date of the LTS studies according to Pauwels et al. [11]. Allowing a relative standard uncertainty for long term stability ($u_{lts,rel}$) of 6% of the certified value, the shelf-life at -70 °C was 18 months for plating on BA and 31 months for plating on SB agar. This is illustrated in Fig. 3 showing the 'u_{lts}-triangle': depending on the chosen shelf-life, u_{lts} decreases or increases. The $u_{lts,rel}$ of 6 % will be included in the uncertainty budget of the material.

The initially predicted shelf-life is an indication for the frequency of measurements needed during stability monitoring of IRMM-355 after release. Stability data obtained after the release will be used to confirm the certified values and to expand the expiry date of the certificate.

BA



SB agar

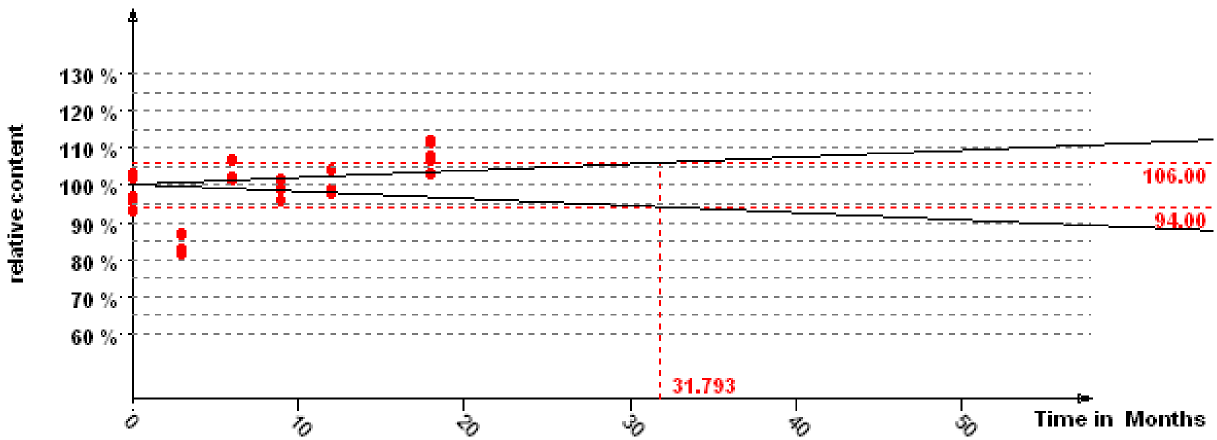


Fig. 3 Graphical representation of shelf-life and u_{lts}- triangle represented by the solid black lines. Allowing a $u_{lts,rel}$ of 6% a shelf-life (storage time) of 18 months (BA) or 31 months (SB agar) was calculated.

7 Batch characterisation

7.1 Design of batch characterisation study

The batch of IRMM-355 was characterised by each of six laboratories by plating and colony counting of 15 material spheres on BA, according to ISO 7218 [8] and 15 material spheres on SB agar, according to ISO 7899-2 [9]. Results of these measurements are summarised in Table 6.

Table 6. Measurement data for batch characterisation of IRMM-355. Individual data for each laboratory (cfu) as well as the mean ($\bar{x}$), standard deviation (*s*) and relative standard deviation (RSD) are shown. The statistically outlying value is marked with *.

A. BA

Vial no	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 1 to 6
1	998	902	755	924	949	887	
2	992	900	724	912	912	817	
3	1000	854	730	914	1063	838	
4	1016	958	694	889	851	843	
5	977	929	664	941	867	850	
6	1012	922	769	885	965	851	
7	983	921	761	944	963	868	
8	1010	844	764	881	977	829	
9	953	931	793	910	950	868	
10	1008	902	816	940	913	823	
11	973	883	731	882	913	864	
12	1023	851	817	916	968	777	
13	961	913	812	930	928	875	
14	934	913	770	999	1056	818	
15	950	856	686	864	811	891	
$\bar{x}$	986	899	752*	915	939	847	**890**
s	27	34	48	34	68	31	81
RSD [%]	2.8	3.8	6.3	3.7	7.2	3.6	9.2

B. SB agar

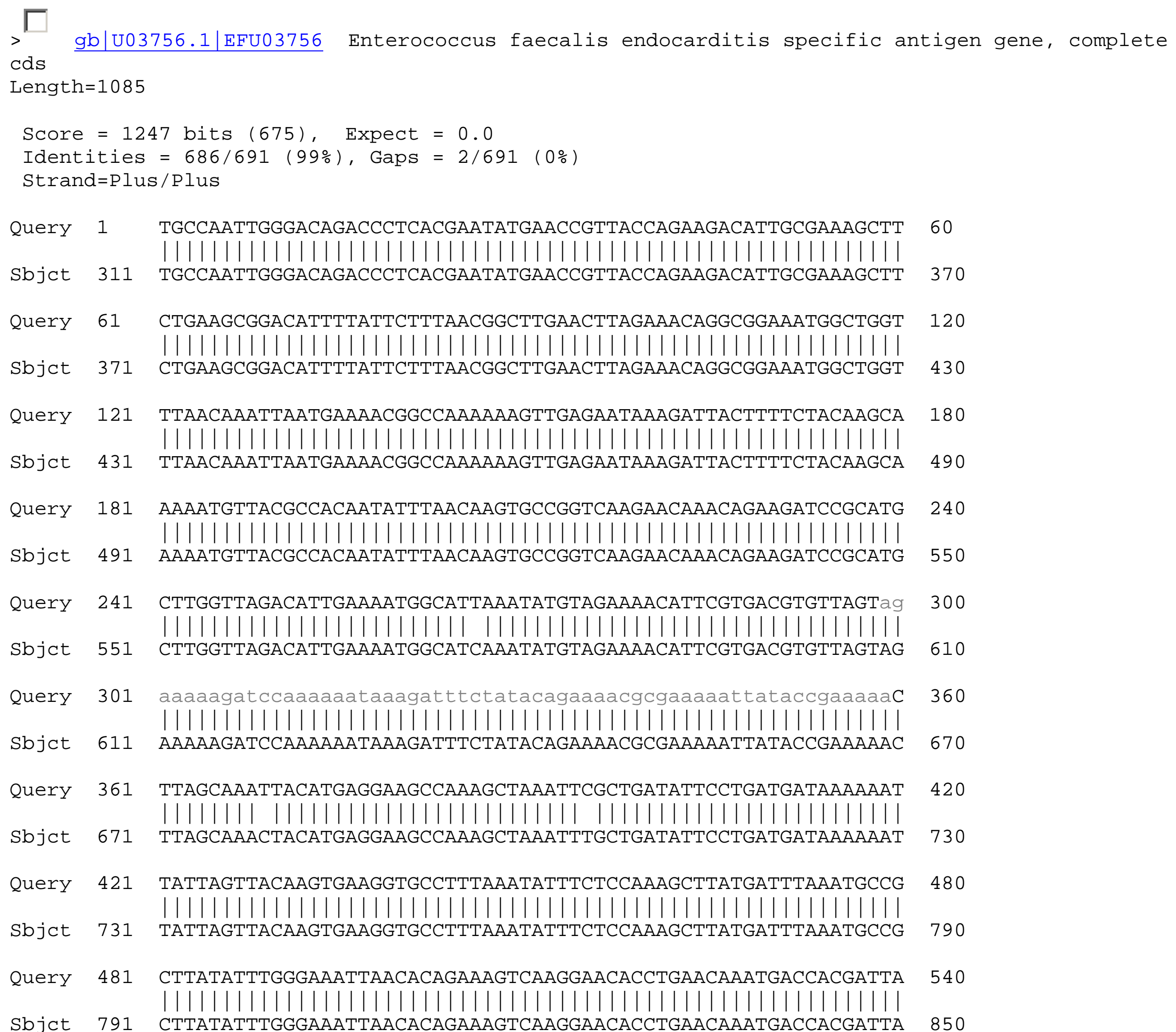
Vial no	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 1 to 6
1	672	847	878	875	869	703	
2	707	822	798	850	856	772	
3	692	855	847	892	900	786	
4	791	855	885	851	915	796	
5	823	826	842	891	851	731	
6	813	844	744	909	886	736	
7	787	770	840	828	918	664	
8	702	880	513	873	862	743	
9	737	830	851	840	891	787	
10	733	854	846	860	932	758	
11	793	834	824	899	885	778	
12	837	843	812	914	857	776	
13	855	819	864	903	903	751	
14	870	809	869	932	894	722	
15	768	849	857	856	883	726	
$\bar{x}$	772	836	818	878	887	749	**823**
s	62	26	91	30	25	36	56
RSD [%]	8.1	3.0	11.2	3.5	2.8	4.8	6.8

7.2 Evaluation of batch characterisation study

For both agars, 6 sets of 15 data points were obtained. The results of the Snedecor F-test and Bartlett test indicate that pooling of data from different laboratories is not allowed and analysis has to be based on laboratory means rather than on individual values. According to the Nalimov *t*-test, the mean cfu value on BA of laboratory 3 is an outlier at $p = 0.05$. Since there was no technical reason to exclude the data, it was decided to retain the dataset of laboratory 3 on BA. The unweighted mean of the laboratory means was used as the certified value i.e. 890 on BA and 823 on SB agar. Since the measurement method does not allow laboratories to report an uncertainty budget, the uncertainty for batch characterisation (u_{char}) was simply calculated as $\frac{s_{means}}{\sqrt{n}}$ with s_{means} being the standard deviation of laboratory means and n being the number of laboratories (Section 9.2).

8 Additional characterisation

The DNA sequence of *efa*A is characteristic for *E. faecalis* and can be used for detection, identification and distinction from other *Enterococcus* strains [10]. Three random samples from the batch were analysed by amplification of the *efa*A gene by PCR using primers *efa*A-f and *efa*A-r. The PCR product was purified and cloned into the vector pCR2.1. Plasmid DNA of pCR2.1 containing the 688 bp amplicon of the *efa*A gene was purified and used for the BigDye® Terminator v1.1 cycle sequencing reaction 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 99 % sequence identity with the reported DNA sequence of the *efa*A gene of *E. faecalis* confirming the identity of the bacteria as *E. faecalis* (Fig. 4).



```
>  gb|U03756.1|EFU03756  Enterococcus faecalis endocarditis specific antigen gene, complete
cds
Length=1085

 Score = 1247 bits (675),  Expect = 0.0
 Identities = 686/691 (99%), Gaps = 2/691 (0%)
 Strand=Plus/Plus

Query  1    TGCCAATTGGGACAGACCCTCACGAATATGAACCGTTACCAGAAGACATTGCGAAAGCTT  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  311  TGCCAATTGGGACAGACCCTCACGAATATGAACCGTTACCAGAAGACATTGCGAAAGCTT  370

Query  61   CTGAAGCGGACATTTTATTCTTTAACGGCTTGAACTTAGAAACAGGCGGAAATGGCTGGT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  371  CTGAAGCGGACATTTTATTCTTTAACGGCTTGAACTTAGAAACAGGCGGAAATGGCTGGT  430

Query  121  TTAACAAATTAATGAAAACGGCCAAAAAAGTTGAGAATAAAGATTACTTTTCTACAAGCA  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  431  TTAACAAATTAATGAAAACGGCCAAAAAAGTTGAGAATAAAGATTACTTTTCTACAAGCA  490

Query  181  AAAATGTTACGCCACAATATTTAACAAGTGCCGGTCAAGAACAAACAGAAGATCCGCATG  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  491  AAAATGTTACGCCACAATATTTAACAAGTGCCGGTCAAGAACAAACAGAAGATCCGCATG  550

Query  241  CTTGGTTAGACATTGAAAATGGCATTAAATATGTAGAAAACATTCGTGACGTGTTAGTag  300
            ||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct  551  CTTGGTTAGACATTGAAAATGGCATCAAATATGTAGAAAACATTCGTGACGTGTTAGTAG  610

Query  301  aaaaagatccaaaaaaataaagatttctatacagaaaacgcgaaaaattataccgaaaaaC  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  611  AAAAAGATCCAAAAAAATAAAGATTTCTATACAGAAAACGCGAAAAATTATACCGAAAAAC  670

Query  361  TTAGCAAATTACATGAGGAAGCCAAAGCTAAATTCGCTGATATTCCTGATGATAAAAAAT  420
            |||||||| ||||||||||||||||||||||||| |||||||||||||||||||||||||
Sbjct  671  TTAGCAAACTACATGAGGAAGCCAAAGCTAAATTTGCTGATATTCCTGATGATAAAAAAT  730

Query  421  TATTAGTTACAAGTGAAGGTGCCTTTAAATATTTCTCCAAAGCTTATGATTTAAATGCCG  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  731  TATTAGTTACAAGTGAAGGTGCCTTTAAATATTTCTCCAAAGCTTATGATTTAAATGCCG  790

Query  481  CTTATATTTGGGAAATTAACACAGAAAGTCAAGGAACACCTGAACAAATGACCACGATTA  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  791  CTTATATTTGGGAAATTAACACAGAAAGTCAAGGAACACCTGAACAAATGACCACGATTA  850
```

```
Query  541   TTGATACCATTAAGAAATCAAAAGCACCTGTGTTATTTGTTGAAACCAGTGTCGATAAAC  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  851   TTGATACCATTAAGAAATCAAAAGCACCTGTGTTATTTGTTGAAACCAGTGTCGATAAAC  910

Query  601   GTAGTATGGAACGGGTCTCAAAAGAAGTGAAAC-GACCAATTTACGATACACTTTTCACA  659
             ||||||||||||||||||||||||||||||||| | ||||||||||||||||||||||||
Sbjct  911   GTAGTATGGAACGGGTCTCAAAAGAAGTGAAACAG-CCAATTTACGATACACTTTTCACA  969

Query  660   GACTCCCTTGCCAAAGAAGGAACAGAAGGCG  690
             |||||||||||||||||||||||||||||||
Sbjct  970   GACTCCCTTGCCAAAGAAGGAACAGAAGGCG  1000
```

Fig. 4 Comparison of the DNA sequence of the *efa*A amplicon obtained from IRMM-355 (Query) with the reported DNA sequence of the *efa*A gene (Genbank accession no. EFU03756) using Nucleotide-nucleotide BLAST (blastn) on www.ncbi.nlm.nih.gov.

9 Certified values and uncertainties

9.1 Certified value

The certified value is calculated as the unweighted mean of the means of the accepted datasets from batch characterisation and is 890 cfu on BA and 823 cfu on SB agar (Table 6).

9.2 Estimation of the uncertainties

The certified expanded uncertainties consist of standard uncertainties related to characterisation (u_{char}), between-bottle homogeneity (u_{bb}) and degradation during long-term storage (u_{lts}).

- No information is available on the uncertainty sources from the participating laboratories. Therefore u_{char} was estimated as $\frac{s_{means}}{\sqrt{n}}$ where s_{means} is the standard deviation of laboratory means and n is the number of laboratories.
- u_{bb} was estimated as the standard deviation between bottles (s_{bb})
- Allowing a relative u_{lts} of 6 % a shelf-life of 18 months for BA and 31 months for SB agar was obtained from stability studies up to 18 months (Section 6.2)

The combined standard uncertainties were calculated as the square root of the sum of squares of the individual contributions, according to:

$$u_c = \sqrt{u_{char}^2 + u_{bb}^2 + u_{lts}^2}$$

The expanded uncertainties U_{CRM} were calculated from the combined standard uncertainty u_c by multiplication with a coverage factor k = 2.

Table 7. Standard uncertainties and expanded uncertainties (k = 2) for IRMM-355 on BA and SB agar.

	u_{char}	u_{bb}	u_{lts}	u_c	U_{CRM}
BA	33	24	53	67	135
SB agar	23	32	49	63	126

10 Metrological traceability

The certified value (cfu per material sphere) is traceable to the SI unit 1 applying procedures ISO 7218 and ISO 7899-2. ISO 7218 [8] provides a procedure for standard plating on a solid medium and ISO 7899-2 [9] describes a procedure for plating and counting of intestinal enterococci using the membrane filtration technique. The sequence identity of the gDNA has been confirmed by BigDye® Terminator v1.1 cycle sequencing of the *efa*A gene [10].

11 Instructions for use

11.1 Dispatch

Dispatch to the customer must be done on dry ice. Upon receipt by the customer, the material can be used immediately or stored at -20 °C or -70 °C. If stored at -20 °C a later temperature shift back to -70 °C should be avoided since less is known about the impact of subsequent storage temperatures on the viability and recovery of the bacterial cells.

11.2 Instructions for use

The bacterial cells in this preparation do not survive at room temperature. Therefore take vials out of the freezer one by one during analysis. Use sterile consumables in all steps and work under sterile conditions.

Horse blood agar (standard plating)

1. Use 10 appropriately conditioned media plates per material sphere.
2. Open vial containing material sphere by aseptically removing the stopper.
3. Add 1 mL of an aqueous solution of NaCl (mass concentration of 0.9 g/L) to the vial. Wait 30 s and homogenise by pipetting up and down.
4. Make sure to remove liquid as much as possible from the vial by distributing 9 portions of 100 µL and one portion containing the rest of the solution on 10 agar plates.
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 5 times lightly up and down on the agar surface of the last plate to help remove any remaining fluid on the spreader.
7. Dry plates for 30 minutes at room temperature before inverting.
8. Incubate at 37 °C ± 1 °C for 14 h to 24 h.

Slanetz and Bartley agar (membrane filtration)

1. Use 20 appropriately conditioned media plates per material sphere.
2. Open vial containing material sphere by aseptically removing the stopper.
3. Add 1 mL of an aqueous solution of NaCl (mass concentration of 0.9 g/L) to the vial. Wait 30 s and homogenise by pipetting up and down.
4. Make sure to remove liquid as much as possible from the vial by distributing 19 portions of 50 µL and one portion containing the rest of the solution over 20 Falcon tubes each containing 10 mL of an aqueous solution of NaCl (mass concentration of 0.9 g/L).
5. Filter the solution in each Falcon tube using a membrane filtration apparatus with a vacuum manifold and filter funnels containing a 47 mm membrane (pore size 0.45 µm):
    - 5a. Pour the solution onto the filter with the suction off
    - 5b. Activate the suction to remove the solution
    - 5c. Rinse the Falcon tube with 10 mL of an aqueous solution of NaCl (mass concentration of 0.9 g/L) and pour it onto the filter with the suction off.
    - 5d. Activate the suction to remove the solution
    - 5e. Repeat the rinsing (2 washes in total)
6. With sterile tweezers place the filter onto an agar plate.
7. Leave plates for 30 minutes at room temperature before inverting.
8. Incubate at 36 °C ± 2 °C for 40 h to 44 h.

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Abstract

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 already offers microbiological reference materials (RMs) with certified colony forming unit (cfu) numbers[1] per capsule covering six different food and water micro-organisms (http://irmm.jrc.ec.europa.eu). Measurement effects, such as Poisson distribution at low cfu counts, encouraged us to consider alternative approaches for development and certification of reference materials. The production of freeze-dried BioBalls™ is not based on aliquoting but rather on transferring a counted number of bacterial or yeast cells into a single BioBall™ with a modified flow cytometer [1]. The IRMM's portfolio of CRMs in the BioBall™ format currently consists of two low cfu materials (IRMM-351, containing *E. coli* O157 and IRMM-352, containing *S. enteritidis*) and one material at a target level of 1000 cfu (IRMM-354, containing *C. albicans*). IRMM currently extends the range of these CRMs to more micro-organisms at a level of 1000 cfu per material sphere for water control laboratories. *Enterococcus faecalis* is a gram-positive, opportunistic bacterium that inhabits the gastrointestinal tracts of humans and many animals. Because of their ability to acquire high-level resistance to antimicrobial agents, enterococci have emerged as nosocomial pathogens worldwide. *Enterococcus faecalis* causes 80 to 90% of human enterococcal infections [2, 3].
This report describes the certification of a batch of 1000 vials (IRMM-355) containing *E. faecalis* at a target level of 1000 cfu per material sphere. Homogeneity and stability of the produced batch were analysed at IRMM following in house procedures applying a quality management system according to ISO Guide 34 [4-7]. Batch characterisation was performed by IRMM and five external laboratories. The methods used for the batch characterisation as well as for homogeneity and stability testing were colony counting on Horse Blood Agar (BA) and Slanetz and Bartley agar (SB agar) according to ISO 7218 [8] and ISO 7899-2 [9] respectively. BA is Columbia agar containing 5 % horse blood and is used as a non-selective growth agar. SB agar is a selective growth agar for the detection and enumeration of intestinal enterococci in surface and waste water.
Identification of *E. faecalis* was performed by DNA sequence analysis of the *efa*A gene encoding the endocarditis antigen [10].

[1] Numbers refer to entitic numbers throughout the whole report

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