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Certification of a reference material with *Candida albicans* (NCPF 3179) at a target level of 1000 colony forming units per material sphere IRMM-354

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IRMM information
REFERENCE MATERIALS

Certification of a reference material with *Candida albicans* (NCPF 3179) at a target level of 1000 colony forming units per material sphere

IRMM-354

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Abstract

This report describes the certification of a reference material (IRMM-354) of *Candida albicans*. 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 of IRMM-354 contains one material sphere of *Candida albicans*. 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 nutrient agar (NA) and Oxytetracyclin-Glucose-Yeast Extract (OGYE) 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 NA and OGYE agar. The certified value for NA is 917 cfu per material sphere with an expanded uncertainty of 168. The certified value for OGYE agar is 912 cfu per material sphere with an expanded uncertainty of 173. In both cases, a coverage factor $k = 2$ is used, corresponding to a level of confidence of about 95 %. The authenticity of the material was confirmed by DNA sequence analysis of the coding region for the *hwp*1 gene.

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Glossary

ANOVA	analysis of variance
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
df	degrees of freedom
dNTP	deoxynucleotide triphosphate
DNA	deoxyribonucleic acid
gDNA	genomic DNA
i	portion of 100 µL
i_{sphere}	number of portions per material sphere
IRMM	Institute for Reference Materials and Measurements
k	coverage factor
l	number of laboratories
LTS	long-term stability
MS_{bb}	mean sum of squares between bottles
MS_{wb}	mean sum of squares within bottles
N	total number of vials in the homogeneity study
n	number of laboratories
NA	nutrient agar
NCPF	national collection of pathogenic fungi
OGYE	Oxytetracyclin-Glucose-Yeast Extract
PCR	polymerase chain reaction
RM	reference material
RSD	relative standard deviation
RSD_{method}	relative standard deviation of the method
s	standard deviation
s_{bb}	standard deviation between bottles
s_r	repeatability standard deviation
u_{bb}	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
$\bar{x}$	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]. In 2008, IRMM released its first CRMs in the BioBall™ format, IRMM-351 containing *E. coli* O157 and IRMM-352 containing *S. enteritidis*, both at a level of 5 cfu per material sphere. 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. *Candida albicans*, one of the waterborne pathogens, is the most common opportunistic fungal pathogen of humans. The microorganism can live as a normal habitant in the body of a significant proportion of individuals. However, it can cause life-threatening systemic infections in immuno-compromised people [2].

This report describes the certification of a batch of 1000 vials (IRMM-354) containing *C. albicans* 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 [3-6]. 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 nutrient agar (NA) and Oxytetracyclin-Glucose-Yeast Extract (OGYE) agar according to ISO 7218 [7] and ISO 13681 [8] respectively. NA is a non-selective growth agar for the cultivation of a wide range of bacteria and yeasts. OGYE is a selective growth agar for the cultivation of yeasts and molds.

Identification of *C. albicans* was performed by DNA sequence analysis of the *hwp*1 gene encoding the hyphal wall protein 1 required for virulence [9].

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

2 Participants

The RM was developed and processed by BTF Pty Ltd. (North Ryde, Australia). Analysis of homogeneity and stability and additional characterisation of IRMM-354 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)
- TNO Quality of Life, Zeist, NL
- Central Veterinary Institute, Lelystad, NL (ISO 9001:2000 certified; 11970-2006-AQ-ROT-RvA)
- Institut Pasteur de Lille, Lille, FR (ISO/IEC 17025 accreditation; Cofrac)
- Food and Consumer Product Safety Authority, Zutphen, NL (ISO/IEC 17025 accreditation; RvA L-104)

3 Processing

IRMM-354 was processed in the BioBall™ format [1]. A modified flow cytometer was used, allowing the sorting and counting of a number of cultured yeast 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 were 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 yeast 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) containing one material sphere were produced. Since droplets are collected in the same liquid nitrogen container before freeze-drying, labelling in the order of manufacturing is not possible. Therefore vial identification number 200 does not correspond to the first produced material sphere.

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 NA according to ISO 7218 [7] and on OGYE agar according to ISO 13681 [8]. One material sphere was added to a tube containing 1 mL of an aqueous solution of NaCl (mass concentration 0.9 g/L). From that, 10 portions *i* of 100 µL (consuming the whole solution) were spread over 10 agar plates by standard plating (NA) or pour plating (OGYE). Plates were incubated at 37 °C ± 1 °C (NA) or at 25 °C ± 1 °C (OGYE) for 36 to 48 h. After incubation, colonies on individual plates were counted. To obtain the cfu number per material sphere, counts of the 10 individual plates were summed up according to equation (1).

$$cfu_{sphere} = \sum_{i=1}^{10} cfu_i \quad (1)$$

4.3 PCR

Each reaction mixture for PCR reactions (25 µL total volume) contained PCR buffer (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 yeast 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; 34 cycles denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, extension at 72 °C for 50 s; incubation at 72 °C for 5 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
Candida albicans	*hwp*1 gene, 1180 bp	CRR-f CRR-r	5'-GTT TTT GCA ACT TCT CTT TGT A-3' 5'-ACA GTT GTA TCA TGT TCA GT-3'	[9]

4.4 DNA sequencing

PCR products (25 µL), amplified with primers CRR-f and CRR-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 NA, according to ISO 7218 [7] and on OGYE agar, according to ISO 13681 [8] (Section 4.2). Since one vial contains only one material sphere which is used as a whole, replicate analysis per vial is not possible (Fig.1).

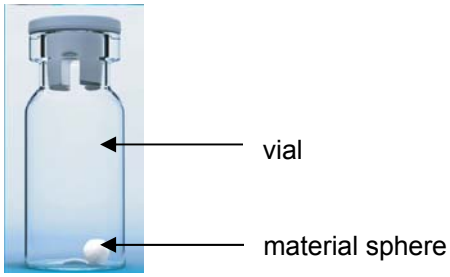


Fig. 1: Representation of a vial of IRMM-354 containing one material sphere. For analysis, a reconstituted material sphere is divided in 10 portions *i*.

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-354. Individual data per vial as well as the mean ($\bar{y}$), standard deviation (*s*) and relative standard deviation (*RSD*) are shown for agars NA and OGYE.

NA		OGYE	
Vial identification number	**Cfu per material sphere**	**Vial identification number**	**Cfu per material sphere**
230	919	**742**	998
279	967	**769**	972
318	1041	**848**	950
391	832	**870**	967
448	1032	**936**	1065
489	1032	**953**	1056
542	1009	**1035**	930
555	1029	**1068**	854
639	982	**1144**	956
655	922	**1198**	847
$\bar{y}$	977	$\bar{y}$	960
s	67.8	***s***	72.2
RSD	7.0 %	***RSD***	7.5 %

The mean of the study was $\bar{y}$ = 977 cfu for colony counting on NA and $\bar{y}$ = 960 cfu for colony counting on OGYE agar. Grubbs tests were performed to detect outlying results. No outliers were detected for NA as well as OGYE agar. Therefore, for both agars, 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 simply the standard deviation of the mean cfu value per material sphere ($\bar{y}$**)** i.e. 67.8 cfu for colony counting on NA and 72.2 cfu for colony counting on OGYE agar. However, to obtain a more realistic estimate for the standard deviation between units, the method repeatability was taken into account.

For this purpose, 5 additional vials were taken randomly from the batch and material spheres were reconstituted in one tube containing 5 mL of an aqueous solution of NaCl (mass concentration 0.9 g/L). The tube was vortexed for 5 seconds resulting in a maximum homogenisation of the content and allowing plating of 49 identical portions *i* of 100 µL. The 50th remaining portion was not taken into account since its volume was not equal to 100 µL. Using this approach, the observed variation of cfu values between plates can only be attributed to the method of plating. The experiment was done for plating and counting on NA as well as on OGYE agar. The resulting RSD_{method} is 10.2 % for NA and 9.8 % for OGYE (Table 3).

Table 3. Measurement data for determination of method repeatability. Individual data per portion as well as the mean value, standard deviation (*s*) and relative standard deviation (*RSD*) are shown for both agars.

	cfu per portion *i*										**mean**	***s***	***RSD*$_{method}$**
NA	104	81	99	84	91	94	111	99	92	99	94.2	9.6	10.2 %
	84	110	98	105	89	82	107	85	91	91			
	101	97	78	102	107	92	67	99	83	95			
	105	96	93	100	84	86	78	88	84	89			
	106	91	96	101	102	101	107	88	103				
OGYE	95	94	104	94	104	115	95	116	104	93	99.8	9.8	9.8 %
	116	88	106	99	80	106	122	109	91	98			
	76	98	99	91	103	102	101	106	102	89			
	95	100	84	106	100	115	94	122	94	109			
	105	94	99	92	89	93	93	105	104				

From this value the repeatability standard deviation (s_r) for the homogeneity study was calculated using equation (2). The relative s_r is 3.2 % for plating on NA and 3.1 % for plating on OGYE agar (Table 4). In a second step, the standard deviation between bottles (s_{bb}) was corrected for the method repeatability using equation (3).

$$s_r = \frac{\sqrt{(RSD_{method})^2 \times i_{sphere}}}{i_{sphere}} \quad (2) \qquad (i_{sphere} \text{ = number of portions per material sphere i.e. 10})$$

$$s_{bb} = \sqrt{s_{\text{hom}}^2 - s_r^2} \quad (3)$$

Table 4 gives values for s_r and s_{bb} on NA and OGYE agar. The value of s_{bb} will be included in the uncertainty budget as an estimate for the uncertainty of between-bottle homogeneity (Section 9.2).

Table 4. Repeatability standard deviation (s_r) and between bottle homogeneity (s_{bb}) for IRMM-354 on NA as well as on OGYE agar

	Relative repeatability standard deviation (s_r)	Relative between bottle homogeneity ($s_{bb, rel}$)	Absolute between bottle homogeneity (s_{bb}) (cfu)
NA	3.2 %	6.2 %	60.57
OGYE	3.1 %	6.8 %	65.28

6 Stability

6.1 Design of stability study

No short term stability study was carried out. It was previously shown for materials IRMM-351 and IRMM-352 that the material spheres are not stable at 4 °C and 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 with time points of 0, 3, 6, 9, 12 and 18 months. For each temperature/time combination three vials were used. 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 yeast cells. Samples were taken from the stock at -70 °C and incubated at -20 °C. At the end of an incubation 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 NA according to ISO 7218 [7] and on OGYE agar according to ISO 13681 [8]. Plates were incubated at 37 °C ± 1 °C (NA) or at 25 °C ± 1 °C (OGYE) for 36 to 48 h.

6.2 Evaluation of stability data

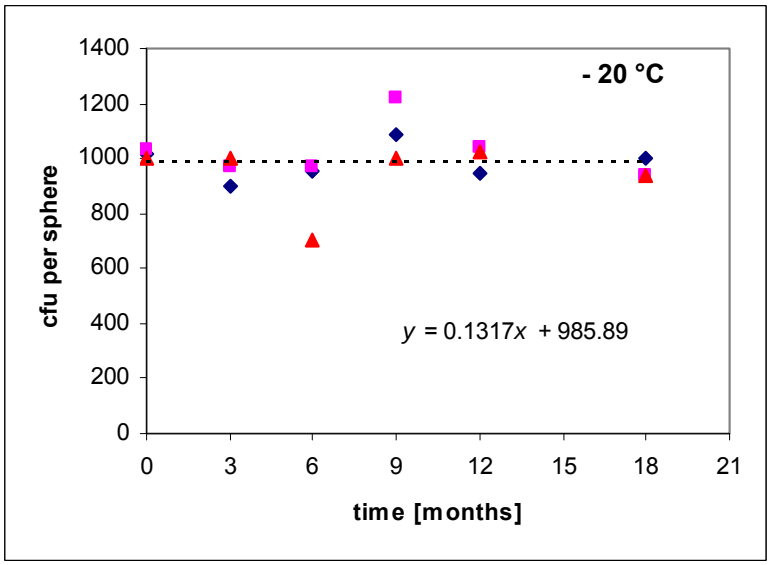
Results of the stability studies are given in Table 5 and plotted in Fig. 2. Three outlying values (marked with a *) were detected using Grubbs tests. However, there is no technical reason for excluding these data.

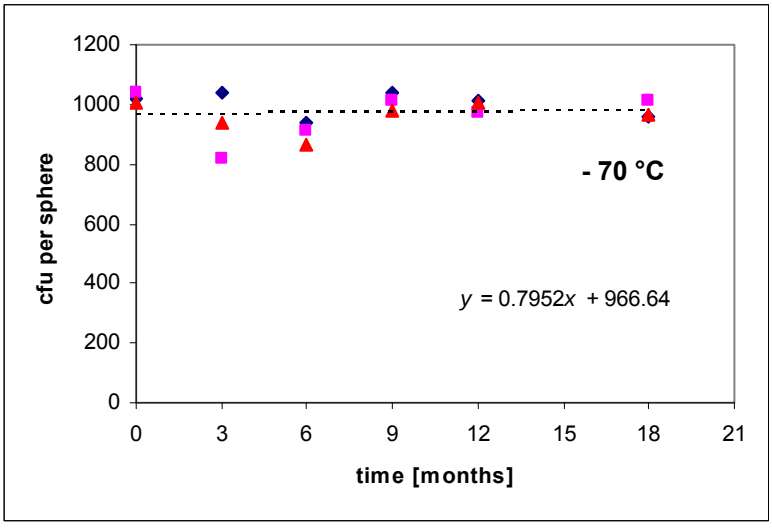
Table 5. LTS data of IRMM-354 stored at two different temperatures. At each temperature/time combination three material spheres were tested. Vials for LTS were taken at random from the batch. *t* = time in months. Outlying values are marked with *.

NA	Cfu per material sphere					
Temperature (°C)	***t* = 0**	***t* = 3**	***t* = 6**	***t* = 9**	***t* = 12**	***t* = 18**
-20	1016	897	956	1091	950	998
-20	1036	972	971	1221	1043	938
-20	1005	1000	703*	1002	1027	939
$\bar{x}$	**1019**	**956**	**877**	**1105**	**1007**	**959**
-70	1016	1038	937	1036	1011	959
-70	1036	815*	909	1013	971	1010
-70	1005	939	866	980	1006	967
$\bar{x}$	**1019**	**931**	**904**	**1010**	**996**	**979**

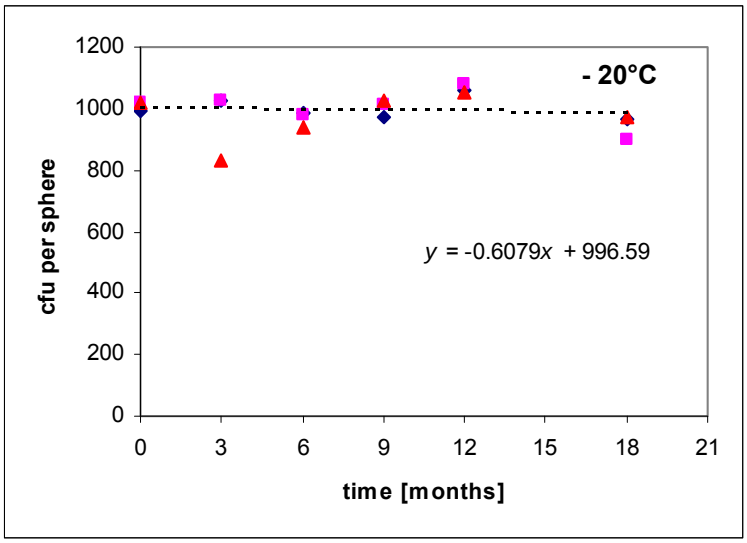
OGYE	Cfu per material sphere					
Temperature (°C)	*t* = 0	*t* = 3	*t* = 6	*t* = 9	*t* = 12	*t* = 18
-20	995	1024	986	972	1056	964
-20	1018	1027	982	1011	1076	899
-20	1022	828*	937	1029	1053	972
$\bar{x}$	**1012**	**960**	**969**	**1004**	**1062**	**945**
-70	995	889	1000	1046	1015	917
-70	1018	975	949	1044	971	984
-70	1022	881	879	974	979	818
$\bar{x}$	**1012**	**915**	**943**	**1022**	**989**	**907**

NA





OGYE



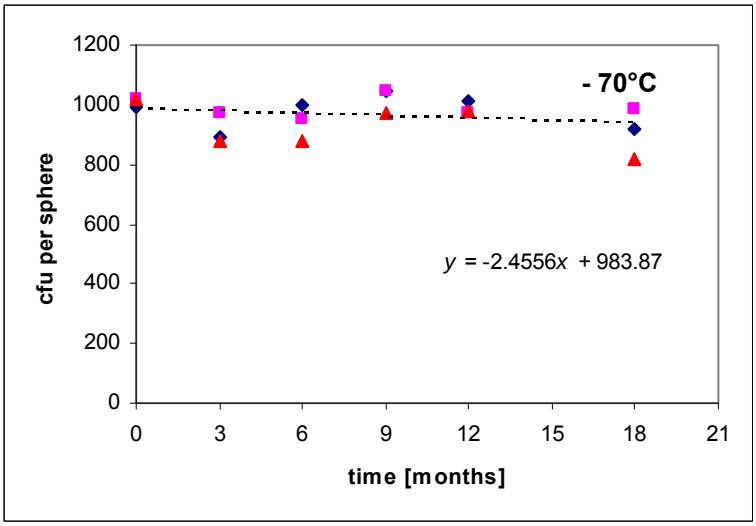


Fig. 2 Graphical representation of LTS data of IRMM-354. At each temperature/time combination three material spheres were tested: ♦, first measurement result; ■, second measurement result ; ▲, third measurement result. The dashed lines represent the calculated regression lines from Table 6. Equations for the regression lines are indicated.

To investigate whether there is a degradation of the material with time, regression lines were calculated and their slopes were tested for significance. For both agars and temperatures, the slopes were insignificant ($|b| / u_b < t_{crit}$) at the investigated level of confidence (Table 6).

Table 6. Test for significance of the slope ($|b| / u_b$) after 18 months storage at the specified temperature.

Temperature [°C]	Agar	slope *b*	intercept *a*	relative u_b	$\|b\| / u_b$	$t_{crit\ (0.05;\ 16)}$
-20	NA	0.13	985.89	4.12	0.03	2.12
-70	NA	0.80	966.64	2.51	0.32	2.12
-20	OGYE	-0.61	996.59	2.45	0.25	2.12
-70	OGYE	-2.46	983.87	2.54	0.97	2.12

The results indicate that it is safe to store the material at -20 °C for 18 months. The shelf-life for storage of the material at -70 °C was calculated according to Pauwels et al. [10]. A shelf-life of 20 months was obtained on NA using a relative uncertainty for long term stability (u_{lts}) of 5%. A slightly lower shelf-life (19 months) was obtained for plating on OGYE. This can be explained by the selective nature of the agar resulting in growth pressure for the yeast cells. The u_{lts} of 5 % will be included in the uncertainty budget of the material.

7 Batch characterisation

7.1 Design of batch characterisation study

The batch of IRMM-354 was characterised by six laboratories by plating and colony counting of 15 material spheres on NA, according to ISO 7218 [7] and 15 material spheres on OGYE, according to ISO 13681 [8]. Results of these measurements are summarised in Table 7.

Table 7. Measurement data for batch characterisation of IRMM-354. Individual data for each laboratory (cfu) as well as the mean ($\bar{x}$), standard deviation (*s*) and relative standard deviation (*RSD*) are shown. Statistically outlying data are marked with *.

A. NA

Vial no	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 1 to 6
1	804	1050	953	978	799	984	
2	801	985	906	997	854	994	
3	868	851	922	961	919	1014	
4	969	850	869	1048	824	1046	
5	651	924	888	1039	877	1010	
6	878	943	982	972	793	1048	
7	912	836	1028	980	658	1040	
8	864	984	992	975	865	1047	
9	834	901	897	1016	898	1051	
10	903	945	910	1026	764	970	
11	717	875	975	861	835	1011	
12	696	946	1000	1025	801	897	
13	925	1140	926	916	813	992	
14	842	1018	930	969	718	982	
15	904	976	929	643	682	1002	
$\bar{x}$	838	948	940	960	807	1006	**917**
s	90.4	82.5	46.0	100.2	75.7	40.5	77.1
***RSD*[%]**	10.8	8.7	4.9	10.4	9.4	4.0	8.4

B. OGYE

Vial no	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 1 to 6
1	827	913	950	982	932	1006	
2	704	1003	900	952	809	1011	
3	874	1040	850	1000	951	1052	
4	870	878	974	1000	881	997	
5	942	932	968	993	685	1054	
6	758	941	975	1021	841	941	
7	757	968	856	739	825	1019	
8	793	872	976	941	913	1023	
9	745	967	976	987	908	996	
10	775	1018	953	974	796	938	
11	650	874	914	892	811	1002	
12	755	25*	892	906	845	1014	
13	819	937	848	1035	900	905	
14	813	893	879	983	848	1013	
15	935	972	923	986	747	1004	
$\bar{x}$	801	943	922	959	846	998	**912**
s	80.7	54.3	48.9	72.2	71.8	40.9	74.1
***RSD*[%]**	10.1	5.8	5.3	7.5	8.5	4.1	8.1

7.2 Evaluation of batch characterisation study

For both agars, 6 sets of 15 data points were obtained. No outlying data were detected except for a value of 25 cfu on OGYE agar reported by Lab 2. According to the laboratory this was most probably due to remaining parts of the matrix in the vial after transferring the material sphere to the tube. Therefore the value of 25 cfu was excluded on a technical basis. Consequently, the instructions for use were slightly changed: an aqueous solution of NaCl (mass concentration 0.9 g/L) is added directly to the vial instead of transferring the material sphere to a tube containing an aqueous solution of NaCl (mass concentration 0.9 g/L).

The results of the Senedecor 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. A Grubbs test was performed to detect outlying laboratory means. No outliers were found and the unweighted mean of the laboratory means was used as the certified value i.e. 917 on NA and 912 on OGYE 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 n being the number of laboratories (Section 9.2).

8 Additional characterisation

The DNA sequence of *hwp*1 is characteristic for *C. albicans* and can be used for detection, identification and discrimination from other *Candida* strains [9]. Three random samples from the batch were analysed by amplification of the *hwp*1 gene by PCR using primers CRR-f and CRR-r. The PCR product was purified and cloned into pCR2.1. Purified plasmid DNA containing the 1180 bp amplicon of the *hwp*1 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 *hwp*1 gene of *Candida albicans* confirming the authenticity of the produced genomic DNA (gDNA) (Fig. 3).

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gb|U64206.1|CAU64206  Candida albicans hyphal wall protein 1 (HWP1) gene, complete
cds
Length=2682


 Score = 2174 bits (1177),  Expect = 0.0
 Identities = 1179/1180 (99%), Gaps = 0/1180 (0%)
 Strand=Plus/Plus

Query  1     GTTTTTGCAACTTCTCTTTGTATCACCTGTATCCGCCTTTTTTAACATAGCAACTCTTGT  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  261   GTTTTTGCAACTTCTCTTTGTATCACCTGTATCCGCCTTTTTTAACATAGCAACTCTTGT  320

Query  61    AAAGTCCCTTTCTTTTCCCACTATTTTATCATTCTTGAAATATGTAATCAGAATAGTTTT  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  321   AAAGTCCCTTTCTTTTCCCACTATTTTATCATTCTTGAAATATGTAATCAGAATAGTTTT  380

Query  121   TCAAAAACTATAAATAACGGTCAAAATAACCGGCTATTTTCAATTTCCATTCAACTTGTT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  381   TCAAAAACTATAAATAACGGTCAAAATAACCGGCTATTTTCAATTTCCATTCAACTTGTT  440

Query  181   TTCTCAACAATATCAAACACAACAGGAATCTCCTATAGTCACTCGCTTTTAGTTTCGTCA  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  441   TTCTCAACAATATCAAACACAACAGGAATCTCCTATAGTCACTCGCTTTTAGTTTCGTCA  500

Query  241   ATATGAGATTATCAACTGCTCAACTTATTGCTATCGCTTATTACATGTTATCAATTGGGG  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  501   ATATGAGATTATCAACTGCTCAACTTATTGCTATCGCTTATTACATGTTATCAATTGGGG  560

Query  301   CCACTGTCCCACAGGTAGACGGTCAAGGTGAAACAGAGGAAGCTCTTATTCAAAAGAGAT  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  561   CCACTGTCCCACAGGTAGACGGTCAAGGTGAAACAGAGGAAGCTCTTATTCAAAAGAGAT  620

Query  361   CTTATGATTACTATCAAGAACCATGTGATGATTACCCACAACAACAACAACAACAAGAGC  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  621   CTTATGATTACTATCAAGAACCATGTGATGATTACCCACAACAACAACAACAACAAGAGC  680

Query  421   cttgtgattacccacaacaacaacaGCAGGAAGAACCTTGTGATTACCCACAACAACAAC  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  681   CTTGTGATTACCCACAACAACAACAGCAGGAAGAACCTTGTGATTACCCACAACAACAAC  740
```

```
Query  481   CACAAGAGCCATGTGACTATCCACAACAGCCACAAGAACCTTGTGACTACCCACAACAAC  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  741   CACAAGAGCCATGTGACTATCCACAACAGCCACAAGAACCTTGTGACTACCCACAACAAC  800

Query  541   CACAAGAACCTTGTGACTACCCACAACAACCACAAGAACCTTGCGACAATCCACCTCAAC  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  801   CACAAGAACCTTGTGACTACCCACAACAACCACAAGAACCTTGCGACAATCCACCTCAAC  860

Query  601   CTGATGTTCCTTGTGACAATCCTCCTCAACCTGATGTTCCTTGTGACAATCCTCCTCAAC  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  861   CTGATGTTCCTTGTGACAATCCTCCTCAACCTGATGTTCCTTGTGACAATCCTCCTCAAC  920

Query  661   CTGATATTCCTTGTGACAATCCTCCTCAACCTGATATTCCTTGTGACAATCCTCCTCAAC  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  921   CTGATATTCCTTGTGACAATCCTCCTCAACCTGATATTCCTTGTGACAATCCTCCTCAAC  980

Query  721   CTGATCAGCCTGATGACAATCCTCCTATTCCAAACATTCCAACCGATTGGATTCCAAATA  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  981   CTGATCAGCCTGATGACAATCCTCCTATTCCAAACATTCCAACCGATTGGATTCCAAATA  1040

Query  781   TTCCAACTGATTGGATCCCAGATATCCCAGAAAAGCCAACAACTCCAGCTACTACTCCAA  840
             |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct  1041  TTCCAACTGATTGGATCCCAGATATTCCAGAAAAGCCAACAACTCCAGCTACTACTCCAA  1100

Query  841   ACATTCCTGCTACAACTACTACTTCTGAATCATCATCTTCTTCTTCTTCTTCATCATCAT  900
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1101  ACATTCCTGCTACAACTACTACTTCTGAATCATCATCTTCTTCTTCTTCTTCATCATCAT  1160

Query  901   ctACTACTCCAAAAACTTCTGCTTCAACTACACCTGAATCTTCTGTTCCAGCTACCACTC  960
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1161  CTACTACTCCAAAAACTTCTGCTTCAACTACACCTGAATCTTCTGTTCCAGCTACCACTC  1220

Query  961   CAAACACTTCTGTTCCAACAACTTCTTCAGAATCAACTACTCCAGCTACTAGCCCAGAAA  1020
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1221  CAAACACTTCTGTTCCAACAACTTCTTCAGAATCAACTACTCCAGCTACTAGCCCAGAAA  1280

Query  1021  GTTCTGTTCCAGTTACTTCTGGATCATCTATTTTAGCTACCACTTCAGAATCATCATCTG  1080
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1281  GTTCTGTTCCAGTTACTTCTGGATCATCTATTTTAGCTACCACTTCAGAATCATCATCTG  1340

Query  1081  CTCCAGCTACTACTCCAAATACATCTGTTCCAACCACTACTACTGAAACCAAATCATCAA  1140
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1341  CTCCAGCTACTACTCCAAATACATCTGTTCCAACCACTACTACTGAAACCAAATCATCAA  1400

Query  1141  GTACTCCATTAACTACTACTACTGAACATGATACAACTGT  1180
             ||||||||||||||||||||||||||||||||||||||||
Sbjct  1401  GTACTCCATTAACTACTACTACTGAACATGATACAACTGT  1440
```

Fig. 3 Comparison of the DNA sequence of the *hwp*1 amplicon obtained from IRMM-354 (Query) with the reported DNA sequence of the *hwp*1 gene (Genbank accession no. U64206) 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 917 cfu on NA and 912 cfu on OGYE agar.

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}) corrected for the repeatability standard deviation (s_r)
- u_{lts} was set at 5% for a shelf-life of 20 months (19 months for OGYE) as estimated from a 18 months stability study (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 8. Standard uncertainties and expanded uncertainties for IRMM-354 on NA and OGYE agar.

	u_{char}	u_{bb}	u_{lts}	u_c	U_{CRM}
NA	31.49	60.57	48.65	83.83	168
OGYE	30.24	65.28	48.22	86.61	173

10 Metrological traceability

The certified value (cfu per material sphere) is traceable to the SI unit 1 applying procedures ISO 7218 and ISO 13681. ISO 7218 [7] provides general rules for microbiological examinations and ISO 13681 [8] describes a colony counting technique for enumeration of yeasts and moulds. The sequence identity of the gDNA has been confirmed by BigDye® Terminator v1.1 cycle sequencing of the *hwp*1 gene [9].

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. No instability of the material was detected when stored at -20 °C or -70 °C for up to 18 months. However, 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 temperature changes on the viability and recovery of the yeast cells.

11.2 Instructions for use

The yeast 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.

1. Use 10 appropriately conditioned media plates per material sphere. For OGYE agar the pour plating technique is used (see below). Use empty Petri dishes and prepare the medium first, according to the instructions of the manufacturer
2. Open vial containing material sphere by aseptically removing the stopper.
3. Add 1 mL of an aqueous solution of NaCl (mass concentration 0.9 g/L) to the vial. Incubate 30 s and homogenise by pipetting up and down.

Nutrient agar (standard plating)

4. Make sure to remove liquid as much as possible from the vial by distributing 10 portions of 100 µL 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 excess fluid on the spreader.
7. Dry plates for 30 minutes at room temperature before inverting.
8. Incubate at 37 °C ± 1 °C for 36 h to 48 h.

Yeast extract, glucose and oxytetracycline/gentamicin (OGYE) Agar (pour plating)

4. Make sure to remove liquid as much as possible from the vial by distributing 10 portions of 100 µL on 10 empty Petri dishes.

5. Add or pour about 15 mL molten (OGYE) Agar, cooled to 45 °C, and swirl immediately to mix well. Swirl each time after adding agar to a plate. Do not wait until agar is added to all plates.

6. Solidify and dry plates for 45 minutes at room temperature before inverting.

7. Incubate at 25 °C ± 1 °C for 36 h to 48 h.

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European Commission

EUR 23707 EN – Joint Research Centre – Institute for Reference Materials and Measurements
Title: Certification of a reference material with *Candida albicans* (NCPF 3179) at a target level of 1000 colony forming units per material sphere.
Author(s): L. De Baets, N. Meeus, H. Schimmel
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Abstract

This report describes the certification of a reference material (IRMM-354) of *Candida albicans*. 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 of IRMM-354 contains one material sphere of *Candida albicans*. 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 nutrient agar (NA) and Oxytetracyclin-Glucose-Yeast Extract (OGYE) 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 NA and OGYE agar. The certified value for NA is 917 cfu per material sphere with an expanded uncertainty of 168. The certified value for OGYE agar is 912 cfu per material sphere with an expanded uncertainty of 173. In both cases, a coverage factor $k = 2$ is used, corresponding to a level of confidence of about 95 %. The authenticity of the material was confirmed by DNA sequence analysis of the coding region for the *hwp*1 gene.

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