European Commission

JRC REFERENCE MATERIALS REPORT

CERTIFICATION REPORT
Certified reference materials for testing of the presence/absence of Staphylococcus aureus enterotoxin A (SEA) in cheese:

IRMM-359a-c

R. Zeleny, J. Charoud-Got, H. Emteborg, H. Schimmel, H. Emons, Y. Nia, I. Mutel, F. Auvray, J.-A. Hennekinne

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Abstract
This report describes the preparation of three cheese powder matrix reference materials (IRMM-359a-c) and their certification for testing of the presence/absence of Staphylococcus aureus enterotoxin A (SEA).
Raw milk cheese was decrusted, cut into cubes, chopped in a kitchen-type food processor for a short time, freeze-dried, cryogenically milled, and mixed (blank material IRMM-359a). Moreover, a second portion of raw milk cheese was decrusted and cut into cubes. After addition of water and spiking with a solution of SEA, the sample was homogenised using a high-speed grinder (Ultra-Turrax). The cheese slurry was freeze-dried, cryogenically milled and mixed with blank cheese powder to obtain the two SEA-containing materials at SEA target levels of 0.1 and 0.25 ng/g cheese, respectively (IRMM-359b, IRMM-359c).
Between unit-homogeneity was quantified and stability during dispatch and storage were assessed in accordance with ISO Guide 35:2006 [1]. The minimum sample intake is 15.1 g cheese powder (representing 25 g of cheese after reconstitution) per replicate analysis (n=5), as stipulated in Commission Regulation 1441/2007 [2], and therefore no dedicated study on the minimum sample intake was performed.
The reference material was characterised in an interlaboratory comparison of laboratories of demonstrated competence and adhering to ISO/IEC 17025 [3] and using the European Screening Method with the VIDAS SET2 and the Ridascreen SET Total for detection (further on named ESM/VIDAS and ESM/Ridascreen, respectively) [4]. Technically invalid results were removed, but no outlier was eliminated on statistical grounds only.
Certified values are reported as probability of detection and expressed as either diagnostic specificity (ratio of true negatives divided by the sum of true negatives and false positives) for the blank material, or diagnostic sensitivity (ration of true positives divided by the sum of true positives and false negatives) for the SEA-containing materials. Uncertainties for homogeneity and stability were estimated, but not used for an uncertainty budget due to the nature of the certified values (presence/absence certification). Instead, the certified values are expressed as intervals with a 95% level of confidence.
The preparation and processing of the material, homogeneity and stability studies, and the characterisation are described hereafter and the results are discussed.

CERTIFICATION REPORT

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Disclaimer

Certain commercial equipment, instruments, and materials are identified in this paper to specify adequately the experimental procedure. In no case does such identification imply recommendation or endorsement by the European Commission, nor does it imply that the material or equipment is necessarily the best available for the purpose.

Summary

This report describes the preparation of three cheese powder matrix reference materials (IRMM-359a-c) and their certification for testing of the presence/absence of *Staphylococcus aureus* enterotoxin A (SEA).

Raw milk cheese was decrusted, cut into cubes, chopped in a kitchen-type food processor for a short time, freeze-dried, cryogenically milled, and mixed (blank material IRMM-359a). Moreover, a second portion of raw milk cheese was decrusted and cut into cubes. After addition of water and spiking with a solution of SEA, the sample was homogenised using a high-speed grinder (Ultra-Turrax). The cheese slurry was freeze-dried, cryogenically milled and mixed with blank cheese powder to obtain the two SEA-containing materials at SEA target levels of 0.1 and 0.25 ng/g cheese, respectively (IRMM-359b, IRMM-359c).

Between unit-homogeneity was quantified and stability during dispatch and storage were assessed in accordance with ISO Guide 35:2006 [1]. The minimum sample intake is 15.1 g cheese powder (representing 25 g of cheese after reconstitution) per replicate analysis (n=5), as stipulated in Commission Regulation 1441/2007 [2], and therefore no dedicated study on the minimum sample intake was performed.

The reference material was characterised in an interlaboratory comparison of laboratories of demonstrated competence and adhering to ISO/IEC 17025 [3] and using the European Screening Method with the VIDAS SET2 and the Ridascreen SET Total for detection (further on named ESM/VIDAS and ESM/Ridascreen, respectively) [4]. Technically invalid results were removed, but no outlier was eliminated on statistical grounds only.

Certified values are reported as probability of detection and expressed as either diagnostic specificity (ratio of true negatives divided by the sum of true negatives and false positives) for the blank material, or diagnostic sensitivity (ration of true positives divided by the sum of true positives and false negatives) for the SEA-containing materials. Uncertainties for homogeneity and stability were estimated, but not used for an uncertainty budget due to the nature of the certified values (presence/absence certification). Instead, the certified values are expressed as intervals with a 95% level of confidence.

The preparation and processing of the material, homogeneity and stability studies, and the characterisation are described hereafter and the results are discussed.

The following values were assigned:

IRMM-359a (blank material):

Blank	Diagnostic specificity [2]	
	Certified value [%] [3]	One-sided lower confidence limit [%] [4]
Staphylococcus aureus enterotoxin A (SEA) [1]	100	97.3

[1] CAS number 642595-84-4. Amino acid sequence as described in: Betley, M.J., Mekalanos, J.J. (1988) Nucleotide sequence of the type A Staphylococcal enterotoxin gene. *J. Bacteriol. 170:* 34-41

[2] As defined in ISO 16140:2003: ratio of true negatives divided by sum of true negatives and false positives.

[3] As determined using the European Screening Method (ESM) with the VIDAS SET2 detection step and the Ridascreen SET Total detection step. The certified value is based on 8 accepted data sets of the ESM with the VIDAS SET 2 detection step and 7 accepted data sets of the ESM with the Ridascreen SET Total detection step. The certified value is traceable to the SI.

[4] The lower confidence limit is based on the results of 15 laboratories. It is determined assuming a Poisson distribution with 112 correct and 0 incorrect results. The value holds for a 95% level of confidence.

IRMM-359b (spiked material, very low level):

Level I	Diagnostic sensitivity [2]	
	Certified value [%] [3]	One-sided lower confidence limit [%] [4]
Staphylococcus aureus enterotoxin A (SEA) [1]	100	97.5

[1] CAS number 642595-84-4. Amino acid sequence as described in: Betley, M.J., Mekalanos, J.J. (1988) Nucleotide sequence of the type A Staphylococcal enterotoxin gene. *J. Bacteriol. 170:* 34-41

[2] As defined in ISO 16140:2003: ratio of true positives divided by sum of true positives and false negatives.

[3] As determined using the European Screening Method (ESM) with the VIDAS SET2 detection step and the Ridascreen SET Total detection step. The certified value is based on 8 accepted data sets of the ESM with the VIDAS SET 2 detection step and 7 accepted data sets of the ESM with the Ridascreen SET Total detection step. The certified value is traceable to the SI.

[4] The lower confidence limit is based on the results of 15 laboratories. It is determined assuming a Poisson distribution with 122 correct and 0 incorrect negative results. The value holds for a 95% level of confidence.

IRMM-359c (spiked material, low level):

Level II	Diagnostic sensitivity [2]	
	Certified value [%] [3]	One-sided lower confidence limit [%] [4]
Staphylococcus aureus enterotoxin A (SEA)[1]	100	97.6

[1] CAS number 642595-84-4. Amino acid sequence as described in: Betley, M.J., Mekalanos, J.J. (1988) Nucleotide sequence of the type A Staphylococcal enterotoxin gene. *J. Bacteriol. 170:* 34-41

[2] As defined in ISO 16140:2003: ratio of true positives divided by sum of true positives and false negatives.

[3] As determined using the European Screening Method (ESM) with the VIDAS SET2 detection step and the Ridascreen SET Total detection step. The certified value is based on 8 accepted data sets of the ESM with the VIDAS SET 2 detection step and 7 accepted data sets of the ESM with the Ridascreen SET Total detection step. The certified value is traceable to the SI.

[4] The lower confidence limit is based on the results of 15 laboratories. It is determined assuming a Poisson distribution with 125 correct and 0 incorrect results. The value holds for a 95% level of confidence.

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Glossary

ANOVA .....Analysis of variances
AU .....Absorbance units
b .....Slope of regression line
BSA.....Bovine serum albumine
CAS.....Chemical Abstracts Services
cKFT .....Coulometric Karl Fischer titration
CPS.....Coagulase-positive Staphylococci
CRM.....Certified reference material
EC .....European Commission
ECDC.....European Center for Disease Prevention and Control
EFSA.....European Food Safety Authority
ELFA .....Enzyme-linked fluorescence assay
ELISA.....Enzyme-linked immunosorbent assay
ESM .....European Screening Method
EU .....European Union
EURL .....European Union Reference Laboratory
FN .....False negative
FP .....False positive
GUM.....Guide to the Expression of Uncertainty in Measurement
IRMM .....Institute for Reference Materials and Measurements
IUB .....International Union of Biochemistry
IUPAC .....International Union for Pure and Applied Chemistry
JRC .....Joint Research Centre of the European Commission
LOD.....Limit of detection
m/m .....Mass-to-mass
$MS_{\text{between-unit}}$.....Mean of squares between units from a 2-way ANOVA
$MS_{\text{within-sample (error)}}$.....Mean of squares within a unit from a 2-way ANOVA
n .....Number of replicates
OD .....Optical density
PBS.....Phosphate buffered saline
PEG .....Polyethylene glycol
PSA.....Particle size analysis
RFV.....Relative fluorescence value
RSD .....Relative standard deviation
RSD_{stab} .....Relative standard deviation of all results of the stability study
s .....Standard deviation
s_{bb}.....Between-bottle standard deviation
SE .....Staphylococcal enterotoxin
SEA.....*Staphylococcus aureus* enterotoxin A
SEB.....*Staphylococcus aureus* enterotoxin B
SEC.....*Staphylococcus aureus* enterotoxin C
SED.....*Staphylococcus aureus* enterotoxin D
SEE.....*Staphylococcus aureus* enterotoxin E
SI .....International Systems of Units
SDS-PAGE.....Sodium dodecyl sulfate polyacrylamide gel electrophoresis
s_{rel}.....relative standard deviation of all results of the stability study
s_{wb} .....Within-bottle standard deviation
$\bar{t}$ .....mean of all t_i
$t_{\alpha,df}$.....Critical t-value for a t-test, with a level of confidence of 1-α, and *df* degrees of freedom
t_i .....Elapsed time at time point i

TN ..... True negative
TP ..... True positive
t_{tt} ..... chosen transport time
t_{sl} ..... chosen shelf life
TV ..... Test value
u^*_{bb} ..... Relative standard uncertainty due to the heterogeneity that can be hidden by the method repeatability
u_{lts} ..... Standard uncertainty of the long-term stability; an additional index "rel" is added as appropriate
u_{sts} ..... Standard uncertainty of the short-term stability; an additional index "rel" is added as appropriate
vKFT ..... Volumetric Karl Fischer titration
$v_{MSwithin}$ ..... Degrees of freedom of MS_{within}
w/w ..... Weight-to-weight

1 Introduction

1.1 Background

Staphylococcal enterotoxins (SEs) released into foods by enterotoxigenic strains of some coagulase-negative but mainly coagulase-positive Staphylococci (CPS), typically *Staphylococcus aureus*, are causative agents for a large number of food-borne illnesses in the EU and elsewhere. For instance, 345 food-borne outbreaks were caused by staphylococcal enterotoxins in the EU in 2011, representing 6 % of all food-borne outbreaks reported in the EU. Foods such as mixed foods (pasta dishes, salads), meat and meat products, egg and egg products, vegetables, baked goods and cheeses were affected [5]. Most outbreaks can be explained by insufficient hygiene practices during processing, cooking or distribution of food products [6-8]. Moreover, insufficient cooling of foods can induce CPS growth and stimulate enterotoxin production, potentially resulting in food poisoning. Typical intoxication symptoms range from nausea to abdominal pain, vomiting, diarrhea, dizziness, and headache [9].

The SEs consist of a family of 22 structurally related proteins with molecular masses of 22-28 kDa. These proteins have shown to be relatively stable to heat treatment, freezing, proteolytic digestion, and changing pH values [10,11].

Depending on the sensitivity of affected individuals, ng to low µg amounts of enterotoxin can cause intoxication with symptoms described above [12]. Therefore, the EU has adopted a legislation to increase consumer protection by defining microbiological criteria for foodstuffs, such as CPS enumeration and SEs detection. In particular, Commission Regulation (EC) No. 2073/2005 [13], amended by Commission Regulation (EC) No 1441/2007 [2] stipulates that in five independent 25 g portions taken from a food sample (milk products such as cheeses, milk powders and whey powders), SEs must not be detected if the food is to be considered safe for human consumption. As analytical method, the so-called European Screening Method (ESM) based on extraction, dialysis concentration and qualitative immunochemical detection has to be applied for analysis. The method targets five SE serotypes, namely SEA, SEB, SEC, SED and SEE, and is not able to distinguish among them [4].

ANSES in its function as the European Union Reference Laboratory (EU RL) for CPS has highlighted the demand to have available suitable RMs for a number of Staphylococcal enterotoxins in food matrices. ANSES and JRC-IRMM agreed to collaborate to establish a reference material for SEA in cheese, currently seen as a priority analyte/matrix combination. SEA, a single-chain 233 amino acid containing 27 kDa protein [14] is the SE serotype most frequently involved in food-borne staphylococcal illnesses [15].

1.2 Choice of the material

Cheese is one of the foods repeatedly associated with Staphylococcal food-poisoning outbreaks. Especially cheeses fabricated from raw (unpasteurised) milk may be contaminated with *Staphylococcus aureus* and/or its metabolites [16,17]. Therefore, a raw cow milk cheese, variety Tomme de Savoie, was chosen as base material. The moderate fat content (28 % fat in total cheese mass) allowed converting the cheese into a powder by freeze-drying, a gentle technique for preserving materials.

Table 1 and Figure 1 define the analyte in IRMM-359. The envisaged target concentrations for SEA in the materials IRMM-359b and IRMM-359c were 0.1 ng/g and 0.25 ng/g, respectively. In addition, a blank cheese powder (IRMM-359a) was produced. The concentration levels of the spiked materials were chosen to have available materials with

SEA mass fractions close to the limit of detection (LOD) and/or in the lower end of the working range of the ESM. The blank material shall serve to determine the method LOD in the laboratory and, together with a solution of pure SEA, can also be used to establish the recovery of the prescribed extraction/dialysis concentration step.

1.3 Development of the CRM

The CRM project was designed in collaboration between IRMM and ANSES. The materials were processed at IRMM after establishment of a suitable procedure for a larger scale production [18]. The certified values were established by an intercomparison of different expert laboratories using the ESM/VIDAS or the ESM/Ridascreen.

1.4 Definition of the analyte

Table 1. Definition of the protein measured in IRMM-359

Trivial name and abbreviation	CAS number	Chemical formula	Molecular mass (Da)*
Staphylococcus aureus enterotoxin A (SEA)	642595-84-4	$C_{1211}H_{1865}N_{323}O_{376}S_4$	27093.3

* ExPasy Protein Parameter Tool, http://web.expasy.org/protparam/ [19] with entry of sequence shown in figure 1

```
1   SEKSEEINEKDLRKKSELQGTALGNLKQIYYYNEKAKTENKESHDQFLQHTILFKGFFTD   60
61  HSWYNDLLVDFDSKDIVDKYKGKKVDLYGAYYGYQCAGGTPNKTACMYGGVTLHDNNRLT  120
121 EEKKVPINLWLDGKQNTVPLETVKTNKKNVTVQELDLQARRYLQEKYNLYNSDVFDGKVQ  180
181 RGLIVFHTSTEPSVNYDLFGAQGQYSNTLLRIYRDNKTINSENMHIDIYLYTS        240
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Fig. 1: Amino acid sequence of mature *Staphylococcus aureus* enterotoxin A (protein precursor with 257 amino acids without the *N*-terminal signal peptide of 24 amino acids which is cleaved off during maturation) [14]. The amino acids are indicated in the one-letter code [20]

2 Participants

2.1 Project management and evaluation

European Commission, Joint Research Centre, Institute for Reference Materials and Measurements, Reference Materials Unit, Geel, BE
(Accreditation to ISO Guide 34 for production of certified reference materials; BELAC, 268-RM)

2.2 Processing

European Commission, Joint Research Centre, Institute for Reference Materials and Measurements, Reference Materials Unit, Geel, BE
(Accreditation to ISO Guide 34 for production of certified reference materials; BELAC, 268-RM)

2.3 Homogeneity and stability measurements

Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail (ANSES), Maisons-Alfort, FR
(Measurements performed under ISO/IEC 17025 accreditation; COFRAC, 1-2246)

2.4 Characterisation

Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail (ANSES), Maisons-Alfort, FR
(Measurements performed under ISO/IEC 17025 accreditation; COFRAC, 1-2246)

Consal S.a.s., Sermide, IT
(Measurements performed under ISO/IEC 17025 accreditation; ACCREDIA, 0580)

Dairy Science Laboratory, Celbridge, IE
(Measurements performed under ISO/IEC 17025 accreditation; INAB, 141T)

Instituut voor Landbouw- en Visserijonderzoek (ILVO), Melle, BE
(Measurements performed under ISO/IEC 17025 accreditation; BELAC, 033-TEST)

Istituto Zooprofilattico Sperimentale del Piemonte, Liguria e Valle D'Aosta, S.C. Controllo alimenti e igiene delle produzioni, Torino, IT
(Measurements performed under ISO/IEC 17025 accreditation; ACCREDIA, 0200)

Laboratorio de Salud Pública y Laboral de Navarra, Pamplona, ES
(Measurements performed under ISO/IEC 17025 accreditation; ENAC, 194/LE404)

Livsmedelsverket, Microbiology Division, Uppsala, SE
(Measurements performed under ISO/IEC 17025 accreditation; SWEDAC, 1457)

Nederlandse Voedsel- en Warenautoriteit (NVWA), Laboratorium voeder- en voedselveiligheid, Wageningen, NL
(Measurements performed under ISO/IEC 17025 accreditation; RvA, L 104)

Österreichische Agentur für Gesundheit und Ernährungssicherheit (AGES), Institut für medizinische Mikrobiologie und Hygiene, Graz, AT
(Measurements performed under ISO/IEC 17025 accreditation; PSID, 179)

R-Biopharm AG, Darmstadt, DE
(Measurements performed under ISO 9001 certification; DAkkS, 019955 QM08)

Rijksinstituut voor Volksgezondheid en Milieu (RIVM), Centrum voor Zoönosen en Omgevingsmicrobiologie, Bilthoven, NL
(Measurements performed under ISO/IEC 17025 accreditation; RvA, L 421)

Veterinærinstituttet, avdeling Bakteriologi, Mat og GMO, Oslo, NO
(Measurements performed under ISO/IEC 17025 accreditation; Norsk Akkreditering, TEST 110)

Wetenschappelijk Instituut Volksgezondheid - Institut scientifique de santé publique (WIV-ISP), Brussel, BE
(Measurements performed under ISO/IEC 17025 accreditation; BELAC, 081-TEST)

3 Material processing and processing control

3.1 Staphylococcus aureus enterotoxin A – purity assessment, preparation of stock solution, determination of protein concentration, and preparation of spiking solutions

Staphylococcus aureus enterotoxin A (SEA), product number AT101, batch number 72610A, was obtained from Toxin Technology, Sarasota, Florida, US. One vial has a nominal content of 1 mg of lyophilised toxin with a purity of at least 95 % (certificate of analysis as provided by the supplier). A stock solution was prepared by adding 1 mL of MilliQ water and vortexing until a clear solution was obtained. This solution was split into aliquots which were frozen at -20 ^{0}C until further use. The SEA purity was verified in-house by SDS-PAGE and silver staining; apart from the band of about 27 kDa, no other bands were visible on the gel, confirming the purity indicated by the provider.

The protein concentration in the solution was determined by amino acid analysis [21]. Briefly, accelerated acidic digestion was applied by using a microwave-assisted digestion and 6 M HCl containing 0.1 % phenol. Thereafter, the liberated amino acids were separated and quantified using isotope dilution reverse-phase liquid chromatography – electrospray ionisation – tandem mass spectrometry. Pure amino acids and their isotopically labelled analogues were used for calibration and as internal standards for accurate quantification, respectively. Calculation of the protein content of the sample was based on the results obtained for the following four amino acids: alanine, valine, isoleucine, and phenylalanine, and the published amino sequence of the protein [14]. Six independent sub-samples of the prepared solution were analysed with this method, and the mean result and its expanded uncertainty (k=2) was 1.011 ± 0.071 mg SEA/g solution.

The following buffer was used as a diluent for preparing the spiking solutions: 10 mM Na_2HPO_4 x 12 H_2O, 145 mM NaCl, titrated to pH 7.3 using HCl; 0.2 w/w % BSA.

For IRMM-359b, the following spiking solution was prepared: first, the stock solution was diluted 1:20 (20 µL stock solution and 380 µL buffer), followed by another dilution 1:50 (200 µL intermediate solution and 9.8 mL buffer). The nominal SEA concentration of this spiking solution was 1 µg/mL.

For IRMM-359c, the following spiking solution was prepared: first, the stock solution was diluted 1:30 (25 µL stock solution and 725 µL buffer), followed by another dilution 1:15 (addition of 10.5 mL buffer to this intermediate solution). The nominal SEA concentration of this spiking solution was 2.22 µg/mL.

3.2 Origin of the starting material

Cheese of the variety Tomme de Savoie was obtained from Coopérative Laitiere de Yenne Porte de Savoie, Yenne, France. This cheese is made from non-pasteurised raw milk and was checked not to contain SEA. For this, a confirmatory ELISA with a sufficiently low LOD (0.003 ng SEA/g cheese) developed at ANSES [22] and further optimised using commercially available antibodies (Toxin Technology) was applied.

The result ("not detected") confirmed the absence of SEA in the starting material.

3.3 Processing

A suitable processing procedure to obtain sufficiently homogeneous and stable cheese powders in large quantities was developed in the frame of a feasibility study. Details are described elsewhere [18].

300 kg of cheese were used as starting amount. First, the rind of the cheese was manually removed. Then the cheese was cut into large cubes and briefly chopped with a large scale food chopper (UM12, Stephan; Hameln, DE). The chopped cheese was freeze-dried in six sub-batches of about 50 kg in an Epsilon 2-100DS freeze-dryer (Martin Christ; Osterode, DE), using a programme developed and optimised during the feasibility study [18]. The dry mass in the cheese was 60.3 m/m % (average value of six sub-batches, as determined by weighing the cheese before and after freeze-drying). The freeze-dried cheese was then milled in a cryogenic mill (Palla VM-KT, Humboldt-Wedag, Köln, DE) and homogenised by three-dimensional mixing (Dyna MIX-CM200, WEB; Basle, CH). The material was put into plastic drums pre-flushed with inert gas, and the tightly closed drums were stored at -20 ^{0}C until filling - blank material IRMM-359a - or further on used for dilution with two spiked cheese powder intermediate materials (see below).

For the preparation of each SEA containing material (IRMM-359b, very low level; IRMM-359c, low level), 2 kg of fresh cheese (decrusted, pre-cut to small cubes, ground) was mixed with 3 L of warm tap water and converted to a creamy slurry using a high-speed grinder (Ultra Turrax DI 25, IKA; Staufen, DE). The mixture was stirred and kept on a warm plate to ensure dispersion of fat and homogeneity.

For IRMM-359b, 9.7 mL spiking solution (1 µg/mL, see above) was added to 5 kg of cheese slurry, followed by vigorous mixing in a high-speed grinder (Ultra Turrax) for approximately 15 min. The theoretical concentration of SEA in the cheese, not taking into account the water which was removed during the sub-sequent freeze-drying step, was calculated as 4.85 ng SEA/g cheese.

For IRMM-359c, 10.9 mL spiking solution (2.22 µg/mL, see above) was added to 5 kg of cheese slurry, followed by vigorous mixing in a high-speed grinder (Ultra Turrax) for approximately 15 min. The theoretical concentration of SEA in the cheese, not taking into account the water, which was removed during the sub-sequent freeze-drying step, was calculated as 12.10 ng SEA/g cheese.

1 L portions of the slurries were poured into metal trays. After freeze-drying in the Epsilon 2-100DS freeze-drier, the materials were cryogenically milled. Thereafter, the materials were sequentially diluted in three steps with blank cheese powder (1:3, 1:4, and 1:4 mass fractions, respectively), and after each dilution, the materials were mixed in a three-dimensional mixer for one hour. The final theoretical concentration of SEA thus amounts to 0.101 ng/g cheese in material IRMM-359b and to 0.252 ng/g cheese in material IRMM-359c. As the materials are lyophilised cheese powders, these values listed above hold for the reconstituted material (cheese powder after addition of water as outlined in section 10.3 and thorough mixing).

The materials were filled in 79 g portions into plastic zip-lock bags by use of an automated filling machine (All Fill, Sandy, UK), and one bag of each material (IRMM-359a, IRMM-359b, IRMM-359c) was put in aluminized pouches which were thermo-sealed using a sealing machine (Magneta 421 MGS Audion; Weesp, NL). The sets were stored at -70 ^{0}C. The amount filled per sachet equals 130.8 g of reconstituted cheese, thus allowing laboratories to perform five independent analyses per sachet and material, taking into account the prescribed sample intake of 25 g of cheese [2].

3.4 Process control

3.4.1 Water content

The water content in the final materials was measured by volumetric Karl Fischer titration (vKFT) [23]. Three units of the batch were chosen using a random stratified sample picking scheme and each sample unit was analysed in triplicate. The determined mean water content and its standard deviation was 2.12 ± 0.10 g/100 g for IRMM-359a, 2.27 ± 0.10 g/100 g for IRMM-359b, and 2.30 ± 0.08 g/100 g for IRMM-359c.

3.4.2 Particle size measurements

Particle size analysis (PSA) was performed using laser diffraction spectrometry. Three sets of the batch were chosen using a random stratified sample-picking scheme and each sample was analysed in duplicate over an interval of 0.5 to 1000 µm using a Helos laser light scattering instrument (Sympatec GmbH System-Partikel-Technik, Clausthal-Zellerfeld, DE). The result for IRMM-359a was an average particle size of 96 µm (90% of particles smaller than 321 µm), for IRMM-359b an average particle size of 93 µm (90% of particles smaller than 305 µm) and for IRMM-359c an average particle size of 98 µm (90% of particles smaller than 340 µm).

4 Description of the European Screening Method (ESM)

In the following, the ESM is described in short. Explanatory notes to the individual steps and more in-depth description can be found in [4].

Sample preparation:

- Weigh 25.0 ± 0.1 g of the representative (mixed) sample into a beaker

Extraction:

- Add 40 mL of warm distilled or osmosis water (38 ± 2 ^{0}C) to the test portion
- Homogenise the sample using a turrax, blender or stomacher. Rinse the system with distilled water.
- Allow the toxin to diffuse by shaking the sample at room temperature for at least 30 min.
- Acidification: add a few drops of HCl (5 M or 1 M as appropriate) to obtain a pH of 3.5 - 4.0. Use pH meter to check pH.
- Centrifuge for 15 min at 3130 x *g* at 4 ^{0}C or room temperature, transfer the supernatant to a beaker.
- Check the pH, it needs to be <4.5. If this is not the case, add HCl to obtain pH of 3.5 - 4.0 and re-centrifuge at the conditions described above.
- Neutralisation: add a few drops of NaOH solution (5 M and 1 M as appropriate) to obtain a pH of 7.4 - 7.6.

Dialysis concentration:

- Prepare a 30 (w/v)% polyethylene glycol (PEG) 20000 solution
- Prepare a 5-6 cm long dialysis membrane (molecular weight cut-off 6000 – 8000 Da, flat width 23 ± 2 mm (e.g. from Spectra/Por®)
- Prepare PBS buffer: 10 mM Na_2HPO_4/145 mM NaCl, pH 7.3 ± 0.2
- Soak membrane according to manufacturer instructions and rinse membrane with distilled water.
- Close one end, load with sample which is first filtered through a funnel with glass wool to remove coarse particles, close second end.
- Put filled membrane in tray with PEG solution and leave overnight at 5 ± 3 ^{0}C.
- Take the dialysis membrane out of the PEG solution and rinse the outer-part of the membrane with distilled water to remove all traces of PEG.
- Add PBS buffer to inner part of membrane and dissolve and recover quantitatively the toxin concentrate; for this, add the buffer in portions, the final mass has to be in the range 5.0 - 5.8 g.
- If the extract is analysed with the VIDAS SET2 detection assay, analysis has to be performed immediately after preparation. In case the Ridascreen SET Total assay is used, store the extract at 5 ± 3 ^{0}C if the analysis is performed within 48 hours; otherwise store the extract at ≤ -18 ^{0}C. Make sure the extract is fully defrosted and homogenised before analysis.

Detection steps – general remarks:

- One important point shall be noted, which holds for both detection steps: the assays target 5 SEs (SEA, SEB, SEC, SED, and SEE); a positive result indicates the presence of any, several, or all of those SEs. Consequently, no discrimination can be made as to which of these five SEs are present.
- It is important that both assays provide quantitative results. However, the results are not expressed in toxin mass fraction (e.g. ng toxin/g matrix), but either as an optical density (OD) in case of the Ridascreen assay or as a test value (TV) in case of the VIDAS assay. The ESM specifies a result at or above the so-called threshold (cut-off) value as "SE(s)- containing sample". Likewise, a result below the threshold (cut-off) is typed "sample does not contain SEs".

VIDAS SET2 detection step:

- The detection is based on an enzyme-linked fluorescence assay (ELFA).
- Use 500 µL of the extract and follow the manufacturer's instructions.
- The kit contains a solution of SEA which is used for one-point calibration of the assay. Details can be found in the instructions for use coming with the kit.
- A negative control and a positive control (contained in the kit) have to be co-analysed with the samples to verify validity of the measurement (automatic interpretation of instrument whether the values for those controls are in a valid interval).
- For each sample, two fluorescence measurements are automatically performed in the cuvette: the first one to establish the background (substrate only), the second one after incubation of the substrate with the enzyme contained in the so-called solid phase receptacle (SPR) device. The difference of those measurements represents the so-called relative fluorescence value (RFV).
- The test value (TV) of the sample is calculated by the automated VIDAS instrument as follows: RFV of the sample divided by RFV of the standard.
- For the VIDAS SET2 detection step, the threshold is fixed at a test value of 0.13 (established during assay validation at the kit provider).

Ridascreeen SET Total detection step

- The detection is based on an enzyme-linked immunosorbent assay (ELISA).
  - Use 100 µL of the extract and follow the manufacturer's instructions. Use a dual wavelength detection at 450/630 nm.
- The absorbance of the positive control shall be higher than or equal to 1.0 (450/630 nm readout with spectrophotometer).
- The absorbance of the negative control shall be lower than or equal to 0.1 (450/630 nm readout with spectrophotometer).
  If one of these controls (positive and negative) does not meet these requirements, the results are considered invalid, and the measurements have to be repeated.
  - For the Ridascreen SET Total detection step, the cut-off value is calculated by adding 0.15 absorbance units (AUs) to the OD-value obtained for the negative control.

Interpretation of the results:
a) VIDAS SET2

The interpretation is as follows: a TV <0.13 indicates a negative result (SE(s) not present or present below LOD of the assay), whereas a TV equal to 0.13 or higher indicates a positive result (SE(s) present in the sample).

b) Ridascreen SET Total assay

The interpretation is as follows: a result below the cut-off indicates a negative result (SE(s) not present or present below LOD of the assay), whereas a result at or above the cut-off indicates a positive result (SE(s) present in the sample).

5 Homogeneity study

A key requirement for any reference material aliquoted into units is the equivalence between those units. In this respect, it is relevant whether the variation between units is significant compared to the uncertainty of the certified value, but it is not relevant if this variation between units is significant compared to the analytical variation. Consequently, ISO Guide 34 [24] requires RM producers to quantify the between unit variation. This aspect is covered in between-unit homogeneity studies.

The within-unit inhomogeneity does not influence the uncertainty of the certified value when the minimum sample intake is respected, but determines the minimum size of an aliquot that is representative for the whole unit.

5.1 Between-unit homogeneity

Only the SEA-containing materials (IRMM-359b, IRMM-359c) were subjected to a homogeneity study. The between-unit homogeneity was estimated as presented below, primarily to have a quantitative assessment of the study, but not used for an uncertainty budget due to the nature of the certified value (presence/absence certification, traditional uncertainty of the certified value not applicable).

The number of units selected corresponds to approximately the cube root of the total number of the units produced. Ten units were selected using a random stratified sampling scheme covering the whole batch for the between-unit homogeneity test. For this, the batch was divided into groups (with a similar number of units) and one unit was selected randomly from each group. Three independent samples were taken from each selected unit, and analysed using the ESM with the VIDAS SET2 detection step as described above [3].

Based on long-time experience with this measurement method at ANSES and as also stipulated in the ESM, extracts were analysed as soon as possible after preparation and not stored longer than a few hours before analysis.

The large number of samples and the time-consuming sample preparation procedure made it necessary to perform the measurements under intermediate precision conditions, whereby on the three measurement days, nine, nine, and twelve extractions with subsequent measurements were performed, respectively. Within each day, samples were analysed in a randomised manner to be able to separate a potential analytical drift from a trend in the filling sequence.

All individual results were correctly reported as "SEs present" for the samples of IRMM-359b and IRMM-359c. Moreover, and this can be considered as an additional homogeneity check, all IRMM-359a, IRMM-359b, and IRMM-359c results from the laboratories during the characterisation study were also correct (all individual IRMM-359a results were reported as "SEs absent", and all individual IRMM-359b and IRMM-359c results were reported as "SEs present"). These results prove that the materials were sufficiently homogeneous.

In addition, the test values obtained for each sample and replicate were evaluated. The results are shown as graphs and tables in Annex A.

Regression analysis was performed to evaluate potential trends in the analytical sequence as well as trends in the filling sequence. No trends in the filling sequence or the analytical sequence were visible for SEA in either material at a 95 % confidence level. The dataset was assessed for consistency using single and double Grubbs outlier tests at a confidence level

of 99 % on the individual results and on the unit means. No outliers were detected for SEA in either material at a 99 % confidence level.

Quantification of between-unit inhomogeneity was undertaken by analysis of variance (ANOVA), which can separate the between-unit variation (s_{bb}) from the within-unit variation (s_{wb}). The latter is equivalent to the method repeatability if the individual samples are representative for the whole unit.

Evaluation by ANOVA requires unit means which follow at least a unimodal distribution and results for each unit that follow unimodal distributions with approximately the same standard deviations. Distribution of the unit means was visually tested using histograms and normal probability plots. Minor deviations from unimodality of the individual values do not significantly affect the estimate of between-unit standard deviations. Individual results were normally distributed, and unit means were unimodally distributed.

It should be noted that $s_{bb,rel}$ and $s_{wb,rel}$ are estimates of the true standard deviations and therefore subject to random fluctuations. Therefore, the mean square between groups ($MS_{between}$) can be smaller than the mean square within groups (MS_{within}), resulting in negative arguments under the square root used for the estimation of the between-unit variation, whereas the true variation cannot be lower than zero. In this case, u^*_{bb}, the maximum inhomogeneity that could be hidden by method repeatability, was calculated as described by Linsinger et al. [25]. u^*_{bb} is comparable to the limit of detection of an analytical method, yielding the maximum inhomogeneity that might be undetected by the given study setup.

Method repeatability ($s_{wb,rel}$), between-unit standard deviation ($s_{bb,rel}$) and $u^*_{bb,rel}$ were calculated as:

$$s_{wb,rel} = \frac{\sqrt{MS_{within}}}{\bar{y}} \qquad \textbf{Equation 1}$$

$$s_{bb,rel} = \frac{\sqrt{\frac{MS_{between} - MS_{within}}{n}}}{\bar{y}} \qquad \textbf{Equation 2}$$

$$u^*_{bb,rel} = \frac{\sqrt{\frac{MS_{within}}{n}} \sqrt[4]{\frac{2}{v_{MSwithin}}}}{\bar{y}} \qquad \textbf{Equation 3}$$

MS_{within} mean square within a unit from an ANOVA

$MS_{between}$ mean squares between-unit from an ANOVA

$\bar{y}$ mean of all results of the homogeneity study

n mean number of replicates per unit

$v_{MSwithin}$ degrees of freedom of MS_{within}

The results of the evaluation of the between-unit variation are summarised in Table 3.

Table 2: Results of the homogeneity study for SEA in IRMM-359

	IRMM-359b	**IRMM-359c**
RSD [%]	6.145	3.753
MS_{within}	0.00653	0.00425
$MS_{between}$	0.00626	0.01090
s_{wb} [%]	6.186	3.078
s_{bb} [%]	n.c.[1)]	2.224
u^*_{bb} [%]	2.008	0.999

[1)] n.c.: cannot be calculated as $MS_{between} < MS_{within}$

The homogeneity study showed no outlying unit means or trends in the filling sequence. Therefore the between-unit standard deviation can be used as estimate of u_{bb}.

5.2 Within-unit homogeneity and minimum sample intake

The sample intake is prescribed in legislation [2] and the ESM [4]. 25 g of cheese have to be used as starting material for toxin extraction and enrichment per replicate analysis (n=5). Therefore, this sample intake was prescribed in the homogeneity and stability studies as well as the characterisation exercise. It shall be noted that the 25 g refer to real samples, i.e. cheese. As the mass loss resulting from freeze-drying of the reference materials was approximately 39 % (see chapter 3), 15.1 g of the cheese powder must be mixed with 9.9 g of water to achieve 25 g of reconstituted cheese for the three CRMs IRMM-359a-c.
In addition, it shall be noted that from the finally prepared 5.0 – 5.8 g sample extract [4], only a small aliquot is used for the analytical step.

6 Stability studies

Stability testing is necessary to establish the conditions for storage (long-term stability) as well as the conditions for dispatch of the materials to the customers (short-term stability). During transport, especially in summer time, temperatures up to 60 °C could be reached and stability under these conditions must be demonstrated, if the samples are to be transported without any additional cooling.

Time, temperature and light were regarded as the most relevant influences on the stability of the materials. Materials are stored and dispatched in the dark, thus eliminating practically the possibility of degradation by light. Therefore, only the influences of time and temperature needed to be investigated.

The stability studies were carried out using an isochronous design [26]. In this approach, samples are stored for a particular length of time at different temperature conditions. Afterwards, the samples are moved to conditions where further degradation can be assumed to be negligible (reference conditions). At the end of the isochronous storage, the samples are analysed simultaneously under repeatability conditions. Analysis of the material (after various exposure times and temperatures) under repeatability conditions greatly improves the sensitivity of the stability tests.

Stability studies were carried out for the materials IRMM-359b and IRMM-359c.

6.1 Short-term stability study

For the short-term stability study, units were stored at 18 °C and 60 °C for 0, 1, 2 and 4 weeks (at each temperature). The reference temperature was set to -70 °C. Two units per storage time and temperature were selected using a random stratified sampling scheme. From each unit, three samples were measured using the ESM/VIDAS as described above.

The large number of samples and the time-consuming sample preparation procedure made it necessary to perform the measurements under intermediate precision conditions, whereby on the five measurement days, nine, nine, nine, nine, and six extractions with subsequent measurements were performed, respectively. Samples were analysed in a randomised sequence to be able to differentiate any potential analytical drift from a trend over storage time. The results of the measurements are shown in Annex B.

In principle, the ESM delivers a result of "SEs present" or "SEs absent". As it was the case in the homogeneity study, all individual results obtained in the stability study (materials IRMM-359b and IRMM-359c) were typed correctly, i.e. SEs present. However, in order to have a quantitative assessment of stability, the test values obtained in the stability study were also evaluated.

The data were evaluated individually for each temperature. The results were screened for outliers using the single and double Grubbs tests at a confidence level of 99 %. No outlying results were found for SEA in both materials IRMM-359b and IRMM-359c.

In addition, the data were evaluated against storage time and regression lines of test values versus time were calculated. The slopes of the regression lines were tested for statistical significance (increase/loss due to shipping conditions). The slopes of the regression lines were not significantly different from zero (95 % confidence level), at both 18 °C and 60 °C.

The material can be dispatched without further precautions under ambient conditions.

6.2 Long-term stability studies

6.2.1 One-year study

For the one-year long-term stability study, units were stored at 4 °C and -20 ^{0}C for 0, 4, 8 and 12 months. The reference temperature was set to -70 °C.

Two units per storage time were selected using a random stratified sampling scheme. From each unit, three samples were measured using the ESM/VIDAS as described above.

The large number of samples and the time-consuming sample preparation procedure made it necessary to perform the measurements under intermediate precision conditions, whereby on the five measurement days, nine, nine, nine, nine, and six extractions with subsequent measurements were performed, respectively. Samples were analysed in a randomised sequence to be able to differentiate any potential analytical drift from a trend over storage time. The results of the measurements are shown in Annex C.

The data were evaluated individually for each temperature. The results were screened for outliers using the single and double Grubbs tests at a confidence level of 99%. For SEA in IRMM-359b, one outlier was found. As no technical reason for this outlier could be found, the result was retained for statistical analysis. For SEA in IRMM-359c, one outlier was found and excluded from further data evaluation for technical reasons (part of sample lost during extraction).

The data were evaluated against storage time and regression lines of test values versus time were calculated. The slopes of the regression lines were tested for statistical significance, (increase/loss due to storage conditions). For SEA in IRMM-359b, a statistically significant trend was obtained at 4 ºC (95 % confidence level). However, this trend can be regarded as technically irrelevant, as it is caused by a statistical outlier (one replicate at time point 12 months), which was not excluded as no technical reason was found to do so. At -20 °C, the slope of the regression line was not significantly different from zero (95 % confidence level). For SEA in IRMM-359c, the slopes of the regression lines were not significantly different from zero (95 % confidence level) at both 4 °C and -20 °C.

The material can therefore be stored at -20 °C.

6.2.2 Two-year study

An isochronous study was prepared. However, seen the excellent results in the one-year study (suitable material stability at 4 and -20 ^{0}C), it was decided to only use time points 0 and 24 months for measurements.

The units were stored at 4 °C and -20 ^{0}C for 0 and 24 months. The reference temperature was set to -70 °C.

Two units per storage time were selected using a random stratified sampling scheme. From each unit, three samples were measured using the ESM/VIDAS as described above.

The large number of samples and the time-consuming sample preparation procedure made it necessary to perform the measurements under intermediate precision conditions, whereby on the three measurement days, six, six, and six extractions with subsequent measurements were performed, respectively. Samples were analysed in a randomised sequence to be able to differentiate any potential analytical drift from a trend over storage time. The results of the measurements are shown in Annex C.

The data were evaluated individually for each temperature. The results were screened for outliers using the single and double Grubbs tests at a confidence level of 99%. No outlier for SEA in IRMM-359b and IRMM-359c was found.

In addition, the data were evaluated against storage time and regression lines of test values versus time were calculated. The slopes of the regression lines were tested for statistical significance, (increase/loss due to storage conditions). For SEA in IRMM-359b and IRMM-359c, the slopes of the regression lines were not significantly different from zero (95 % confidence level) at both 4 °C and -20 °C.

It was confirmed that the material can be stored at -20 °C.

6.3 Statistical evaluation

Due to the intrinsic variation of measurement results, no study can rule out degradation of materials completely, even in the absence of statistically significant trends. It is therefore necessary to quantify the potential degradation that could be hidden by the method repeatability, i.e. to estimate the uncertainty of stability. This means that, even under ideal conditions, the outcome of a stability study can only be that there is no detectable degradation within an uncertainty to be estimated.

It shall be noted that the relative slopes and ratios of slopes divided by its errors were calculated to test for the significance of a potential trend. The uncertainties of stability during dispatch and storage were estimated, as described in [27]. In this approach, the uncertainty of the linear regression line with a slope of zero was calculated. The uncertainty contributions u_{sts} and u_{lts} were calculated as the product of the chosen transport time/shelf life and the uncertainty of the regression lines as:

$$u_{sts,rel} = \frac{s_{rel}}{\sqrt{\sum \left(t_i - \bar{t}\right)^2}} \cdot t_{tt} \qquad \textbf{Equation 4}$$

$$u_{lts,rel} = \frac{s_{rel}}{\sqrt{\sum \left(t_i - \bar{t}\right)^2}} \cdot t_{sl} \qquad \textbf{Equation 5}$$

s_{rel} relative standard deviation of all results of the stability study
t_i time elapsed at time point *i*
$\bar{t}$ mean of all t_i
t_{tt} chosen transport time (1 week)
t_{sl} chosen shelf life (12 months)

The results of these evaluations are summarised in Tables 3-5.

Table 3. Short-term stability results for IRMM-359b and IRMM-359c

	IRMM-359b		IRMM-359c	
Statistical parameters	18 ºC	60 ºC	18 ºC	60 ºC
Slope (*b*) [%/week]	-0.145	-1.397	0.438	0.509
$\|b\|/s_b$	0.183	0.945	1.010	1.291
Statistical significance (95% conf. interval) [1]	no	no	no	no
u_{sts} [%/week]	0.775	1.475	0.434	0.399

[1] $t_{0.05;22}$= 2.074

Table 4. One-year long-term stability results for IRMM-359b and IRMM-359c

	IRMM-359b		IRMM-359c	
Statistical parameters	4 ºC	-20 ºC	4 ºC	-20 ºC
Slope (*b*) [%/year]	-8.686	-5.785	-0.218	-2.293
$\|b\|/s_b$	2.350	1.505	0.138	1.379
Statistical significance (95% conf. interval) [1]	yes	no	no	no
u_{lts} [%/year]	6.234	3.945	1.545	1.697

[1] $t_{0.05;22}$= 2.074

Table 5. Two-year long-term stability results for IRMM-359b and IRMM-359c

	IRMM-359b		IRMM-359c	
Statistical parameters	4 ºC	-20 ºC	4 ºC	-20 ºC
Slope (*b*) [%/year]	0.303	-2.032	-0.235	0.422
$\|b\|/s_b$	0.131	1.279	0.275	0.539
Statistical significance (95% conf. interval) [1]	no	no	no	no
u_{lts} [%/year]	2.213	1.631	0.820	0.758

[1] $t_{0.05;12}$= 2.179

It shall be noted that the uncertainties were estimated for having quantitative information as concerns stability, but not used later due to the nature of the certified value (presence/absence certification).

After the certification study campaign, the material will be included in IRMM's regular stability monitoring programme to control its further stability.

7 Characterisation

The material characterisation is the process of determining the certified value of a reference material.

This was based on an interlaboratory comparison of expert laboratories. The presence/absence of SEA in the materials was determined in different laboratories which all had to adhere to the official ESM [4]. Consequently, the measurand is operationally defined (defined by the method).

7.1 Selection of participants

Laboratories were selected by IRMM based on criteria that comprised both technical competence and quality management aspects. Each participant was required to operate a quality system and to deliver documented evidence of its laboratory proficiency in detection of SEs in food matrices using the ESM, by submitting results of intercomparison exercises and/or method validation reports. Having a formal accreditation was not mandatory, but meeting the requirements of ISO/IEC 17025 was obligatory. Where measurements are covered by the scope of accreditation, the accreditation number is stated in the list of participants (Section 2).

7.2 Study setup

Each laboratory received the following samples: 3 sets of IRMM-359 (one set consists of one unit each of IRMM-359a, IRMM-359b, and IRMM-359c). The sets with the units for material characterisation were selected using a random stratified sampling scheme and covered the whole batch. Sample preparations and measurements had to be spread over three days to ensure intermediate precision conditions. Laboratories had to follow the technical specifications that were provided together with the samples. In particular, the reconstitution of the cheese powder had to be performed as stipulated in those specifications. Moreover, the ESM had to be strictly followed (accuracy of sample intake, pH adjustments during sample preparation to specified intervals, final mass of extract, etc.).

Three independent sub-samples of each vial had to be prepared and analysed, amounting to nine analyses per material; thus, in total, 27 measurements were performed in each laboratory (54 measurements in case the laboratory used both detection steps).

Reconstitution of the samples was prescribed by IRMM and was performed as follows: 9.9 g of distilled water was to be added to 15.1 g powder. The sample was then to be homogenised by adding a magnetic stirring bar to each powder/water mixture, and stirred for 10-15 min at room temperature. Thereafter, the protocol stipulated in the ESM had to be strictly followed.

In total, 13 laboratories participated in the characterisation study: six laboratories used the ESM/VIDAS, five laboratories used the ESM/Ridascreen, and two laboratories applied both ESM/VIDAS and ESM/Ridascreen. For those two laboratories, it shall be noted that independent samples were prepared for analysis using either detection step (i.e. one extract was not split and analysed with both detection steps). Thus, 15 data sets were received.

7.3 Evaluation of results

The characterisation study resulted in a total of 15 data sets, 8 using ESM/VIDAS and 7 using ESM/Ridascreen. All results of the participants are shown in the graphs and tables in Annex D.

7.3.1 **Technical evaluation**

The obtained data were first checked for compliance with the requested analysis protocol and for their validity based on technical reasons. The following criteria were considered during the evaluation:

- Adherence to the prescribed masses of cheese powder and water to be used for reconstitution.
- pH value after acidification of the sample to be in the interval 3.5 - 4.0;
- pH value after centrifugation to be <4.5 in the supernatant
- pH value after neutralisation to be in the interval 7.4 - 7.6,
- final mass of the sample to be in the interval 5.0 - 5.8 g.

Table 6: Datasets that showed non-compliances with the analysis protocol and technical specifications, and action taken.

	Lab code	Description of problem	Action taken
IRMM-359a	B	3 results of day 1 rejected, as reconstitution was not performed as prescribed (mass of water outside indicated range). Also, one of the 3 results of day 2 rejected due to same issue.	not used for evaluation
IRMM-359b	B	3 results of day 1 rejected, as reconstitution was not performed as prescribed (mass of water outside indicated range). Also, the second and third replicate of day 2 rejected as the final mass was too low (below 5.0 g). Finally, one of the three results of day 3 rejected as reconstitution was not performed as prescribed (mass of water outside indicated range).	not used for evaluation
IRMM-359c	B	One of three results of day 2 rejected, as reconstitution was not performed as prescribed (mass of water outside indicated range).	not used for evaluation
IRMM-359a	C	8 results rejected as pH after neutralisation of the sample was below the stipulated range of 7.4-7.6	not used for evaluation
IRMM-359a	G	One of three results of day 2 rejected, as final mass was outside the prescribed range (5.0- 5.8 g). Also, two of the three results of day 3 were rejected for the same reason.	not used for evaluation
IRMM-359c	G	One of three results of day 1 rejected, as final mass was outside the prescribed range (5.0- 5.8 g)	not used for evaluation
IRMM-359c	J	On day 1, part of the solution was lost when transferring the sample	not used for evaluation
IRMM-359a	K	1 of 9 results retained, as in 8 cases the pH after acidification was above the prescribed range 3.5-4.0	not used for evaluation
IRMM-359b	K	2 of 9 results retained, as in 7 cases the pH after acidification was above the prescribed range 3.5-4.0	not used for evaluation
IRMM-359c	K	2 of 9 results retained, as in 7 cases the pH after acidification was above the prescribed range 3.5-4.0	not used for evaluation

It shall be noted that all rejected results (Table 6) were nevertheless correct (IRMM-359a results reported as "SEs not present", IRMM-359b and IRMM359c results reported as "SEs present").

7.3.2 Statistical evaluation of the results concluded from the ESM measurements

Although quantity values are obtained (so-called Test Values with the VIDAS SET2, OD values expressed as AU with the Ridascreen SET Total), results are expressed as either "SEs absent" (if value below the assay threshold/cut-off) or as "SEs present" (if value above the assay threshold/cut-off). The following results were obtained:

IRMM-359a, 112 valid results, all classified as "absence of SEs"
IRMM-359b, 122 valid results, all classified as "presence of SEs"
IRMM-359c, 125 valid results, all classified as "presence of SEs"

7.3.3 Statistical evaluation of Test Values (VIDAS) and OD values (Ridascreen)

It shall be noted that these values are only used for additional material information and not for value assignment of certified values.

The datasets accepted based on technical reasons were tested for normality of dataset means using kurtosis/skewness tests and normal probability plots and were tested for outlying means using the Grubbs test and using the Cochran test for outlying standard deviations (both at a 99 % confidence level). Standard deviations within (s_{within}) and between ($s_{between}$) laboratories were calculated using one-way ANOVA. The results of these evaluations are shown in Tables 8 (Ridascreen data) and 9 (VIDAS data).

Table 7: Statistical evaluation of the technically accepted datasets for IRMM-359a, SEA in cheese, using the ESM with the Ridascreen SET Total detection step. *p*: number of technically valid datasets

	p	Outliers	Normally distributed	Statistical parameters			
		Means		mean [mg/kg]	*s* [mg/kg]	$s_{between}$ [mg/kg]	s_{within} [mg/kg]
SEA in IRMM-359a	7	none	yes	0.07551	0.019251	0.01481	0.03613
SEA in IRMM-359b	7	none	yes	0.60919	0.15481	0.15446	0.10731
SEA in IRMM-359c	7	none	yes	1.35571	0.36653	0.35573	0.22568

Table 8: Statistical evaluation of the technically accepted datasets for IRMM-359a, SEA in cheese, using the ESM with the VIDAS SET2 detection step. *p*: number of technically valid datasets

	p	Outliers	Normally distributed	Statistical parameters			
		Means		mean [mg/kg]	*s* [mg/kg]	$s_{between}$ [mg/kg]	s_{within} [mg/kg]
SEA in IRMM-359a	8	none	yes	0.00972	0.00566	0.00373	0.00753
SEA in IRMM-359b	8	none	yes	1.13451	0.22802	0.18778	0.15888
SEA in IRMM-359c	8	none	yes	1.96696	0.25853	0.26091	0.13468

8 Value assignment

8.1 Certified values

The method (ESM/VIDAS and ESM/Ridascreen) is a so-called qualitative one. A qualitative method is defined as method of analysis whose response is either the presence or the absence of the analyte, detected either directly or indirectly in a certain amount of sample [28]. The border between "present" (i.e. detected) and "absent" (i.e. not detected) is defined by the threshold value/cut-off of the method.

The certified value has the meaning of a detection probability, expressed as either diagnostic specificity (blank material) or diagnostic sensitivity (SEA-containing materials). These parameters are widely used in food and clinical microbiology [29-31] as well as analytical toxicology [32] to describe performance characteristics of assays applied in these fields. The parameters are defined as follows:

$$Specificity = \frac{TN}{TN + FP} \times 100 \qquad Sensitivity = \frac{TP}{TP + FN} \times 100$$ **Equations 6, 7**

with TN, true negative, TP, true positive, FN, false negative, and FP, false positive. TN and TP refer to correct test results for a given sample (positive sample correctly reported as positive by the assay, negative sample correctly reported as negative by the assay), whereas FN and FP refer to incorrect test results for a given sample (positive sample reported as negative with the assay, negative sample falsely reported as positive with the assay).

In essence, the meaning of the parameters is the following: specificity is the ability of the method to report "not detected" when the analyte is absent in the sample, as indicated by percentage of negative samples reported as negatives. Sensitivity is the ability of the method to detect the analyte when it is present in the sample, as indicated by percentage of positive samples reported as positives

The following certified values were calculated:

IRMM-359a. 112 TN results, 0 FP results, thus specificity of 100 %. The lower limit of confidence was calculated assuming a Poisson distribution as described elsewhere [33]. Using the ESM [4] and IRMM-359a, the probability of obtaining a correct result was 100 %, with a confidence interval ranging from 97.3 % to 100 % at the 95 % confidence level (α= 0.05; n = 112).

IRMM-359b. 122 TP results, 0 FN results, thus sensitivity of 100 %. The lower limit of confidence was calculated assuming a Poisson distribution as described elsewhere [33]. Using the ESM [4] and IRMM-359b, the probability of obtaining a correct result was 100 %, with a confidence interval ranging from 97.5 % to 100 % at the 95 % confidence level (α = 0.05; n = 122).

IRMM-359c. 125 TP results, 0 FN results, thus sensitivity of 100 %. The lower limit of confidence was calculated assuming a Poisson distribution as described elsewhere [33]. Using the ESM [4] and IRMM-359c, the probability of obtaining a correct result was 100 %, with a confidence interval ranging from 97.6 % to 100 % at the 95 % confidence level (α = 0.05; n = 125).

8.2 Additional material information

These values are not certified, but are given as additional material information (Table 9).

Table 9. Additional material information values as obtained in the characterisation study

ESM/VIDAS SET2		
	Test value	
	Mean value[1)]	Interval[2)]
SEA in IRMM-359a	0.01	0.00 – 0.05
SEA in IRMM-359b	1.14	0.47 – 1.53
SEA in IRMM-359c	1.97	1.10 – 2.42
ESM/Ridascreen SET Total		
	Absorbance units	
	Mean value[1)]	Interval[2)]
SEA in IRMM-359a	0.08	0.01 – 0.19
SEA in IRMM-359b	0.61	0.28 – 1.11
SEA in IRMM-359c	1.36	0.45 – 2.31

[1)] mean of mean of 8 data sets (ESM/VIDAS) and 7 data sets (ESM/Ridascreen)
[2)] interval (lowest and highest individual value)

9 Metrological traceability and commutability

9.1 Metrological traceability

The measurement results for assigning a value for the presence of SEA in the material were generated by adhering to the ESM [4]. The sample preparation protocol for extraction and dialysis concentration as well as the analytical detection step with a commercial assay had to be strictly followed. Therefore, the measured properties are so-called operationally defined.

The identity of SEA was assessed by SDS-PAGE (molecular mass deduced from gel) and confirmatory ELISA (SEA-specific).

Traceability of the obtained results is based on the traceability of all relevant input factors. Instruments in individual laboratories were verified and calibrated with tools ensuring traceability to the International System of Units (SI). Consistency in the interlaboratory comparison demonstrates that all relevant input factors were covered. As the assigned values are combinations of agreeing results individually traceable to the SI, the assigned values themselves are traceable to the SI as well.

9.2 Commutability

A dedicated commutability study was not performed for two reasons: firstly, naturally contaminated cheese samples at relevant SEA levels are not available, and secondly, only one method was used for testing the samples. However, in the course of the feasibility study for producing larger batches of cheese powder materials, it was found that cheese powder spiked before analysis at 0.1 and 0.25 ng/g, respectively, and SEA-containing reference materials behaved in a similar way with regard to extraction recovery and results, with recoveries around 50 % [18]. Moreover, experiments performed at ANSES with fresh cheese spiked at the levels indicated above also revealed similar recoveries. These data indicate that the materials behave in the same way in the ESM analytical process as fresh cheese samples.

10 Instructions for use

10.1 Safety precautions

The usual laboratory safety precautions apply.

10.2 Storage conditions

The materials should be stored at a temperature of -20 ± 5 °C. Please note that the European Commission cannot be held responsible for changes that happen during storage of the material at the customer's premises, especially of open samples.

10.3 Reconstitution of the material

- Allow the sachet to warm up to ambient temperature; shake vigorously for at least 30 s before opening.
- Accurately weigh an aliquot of 15.1 ± 0.1 g immediately after opening the sachet to minimise water uptake of the lyophilised powder.
- Add an accurately weighed 9.9 ± 0.1 g of distilled water to the powder.
- The sample must then be homogenised by adding a magnetic stirring bar to the powder/water mixture and stirring for 10-15 min at room temperature. A highly viscous and clumpy homogenate is typically obtained. Once this stage is reached, the instructions in the ESM are to be followed, i.e. addition of 40 g warm water and homogenisation to a slurry by use of a turrax, blender, or stomacher device [4].

10.4 Minimum sample intake

The minimum sample intake is 15.1 g cheese powder (representing 25 g of cheese after reconstitution) per replicate analysis (n=5), as stipulated in Comission Regulation 1441/2007 [2].

10.5 Use of the certified value

This material is intended to be used for method performance control and validation purposes. A laboratory using these CRMs for analyses must compare the results they generate with the certified values (absence of SEA in IRMM-359a, presence of SEA in IRMM-359b and IRMM-359c). Furthermore, the laboratory can compare the quantity values obtained from the ESM/VIDAS and the ESM/Ridascreen (Test Value; OD) with those listed as additional material information on the certificates of the three materials.

11 Acknowledgements

The authors would like to thank Julia Kuhlmann and Blagica Dimitrievska (IRMM, BE) for internal review of this report, as well as the experts of the Certification Advisory Panel "Biological Macromolecules and Biological/Biochemical Parameters", Hez Hird (Food and Environmental Research Agency, UK), Martin Wagner (University for Veterinary Medicine Vienna, AT) and Lothar Siekmann (University of Bonn, DE) for their constructive comments. The authors are grateful for the active involvement of the participating laboratories in the characterisation study.

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13 Annexes

Annex A. Homogeneity data

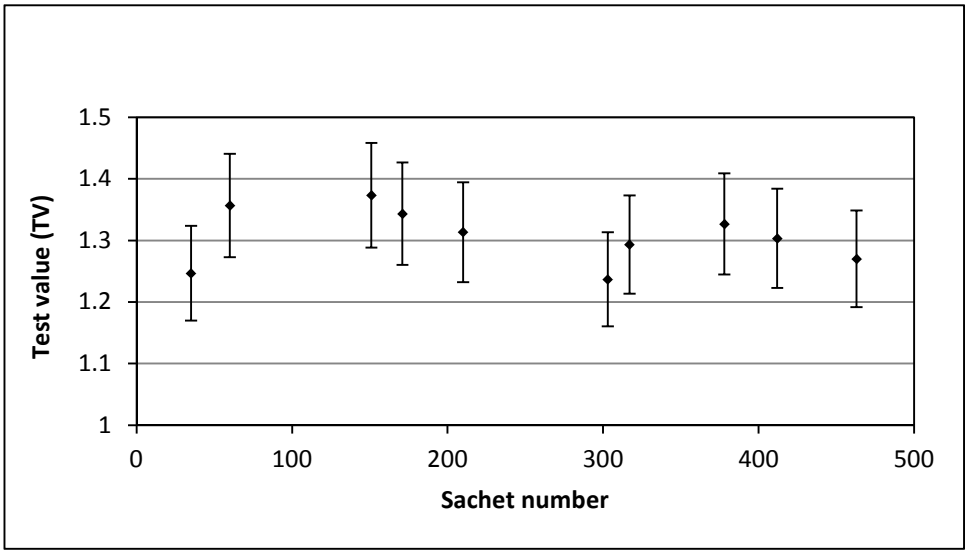

Figure A1. Results of the homogeneity measurements (IRMM-359b). Unit means and their confidence intervals (95 %; n=3) based on the within-group standard deviation derived by ANOVA are shown.

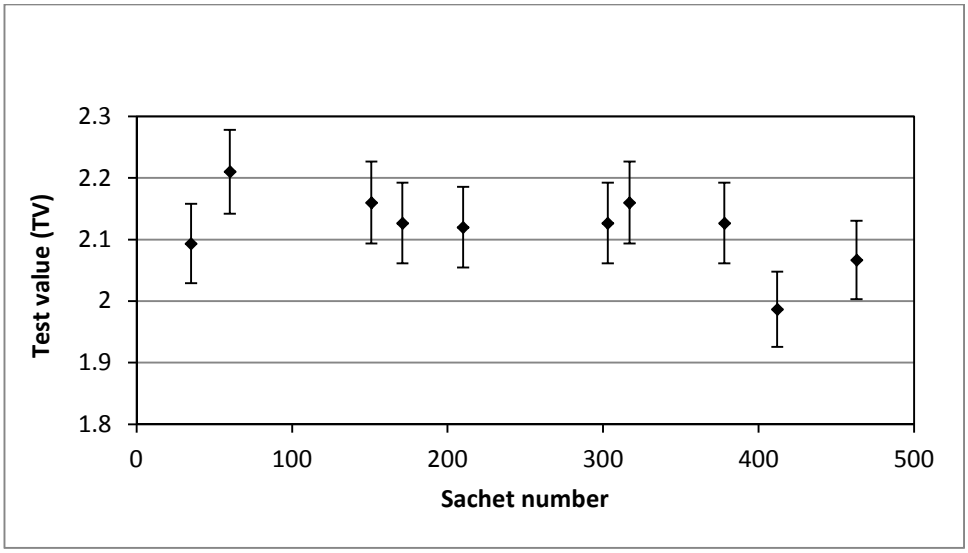

Figure A2. Results of the homogeneity measurements (IRMM-359c). Unit means and their confidence intervals (95 %; n=3) based on the within-group standard deviation derived by ANOVA are shown.

Annex B. Short-term stability data

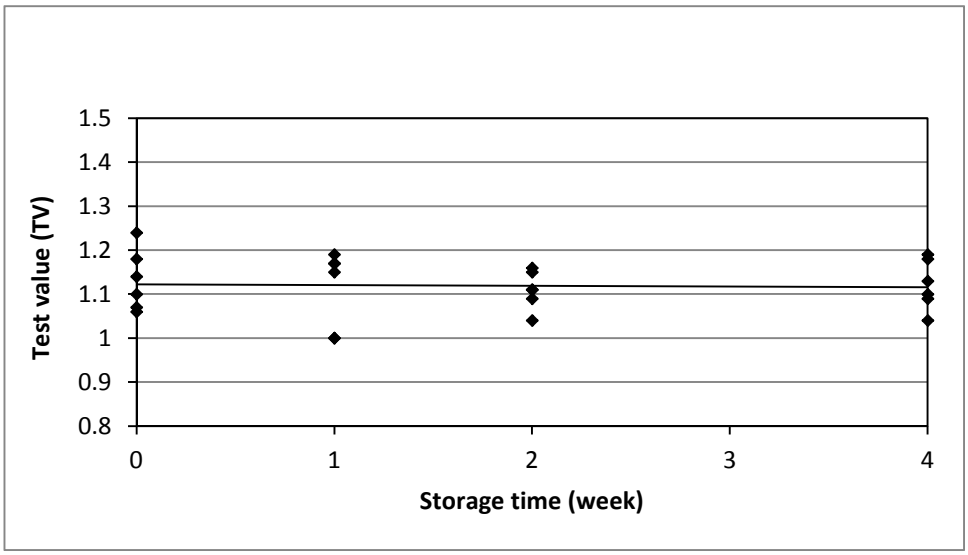

Figure B1. Results of the short-term stability measurements (IRMM-359b, 18 ºC). The obtained results per individual time point and the respective regression line are shown.

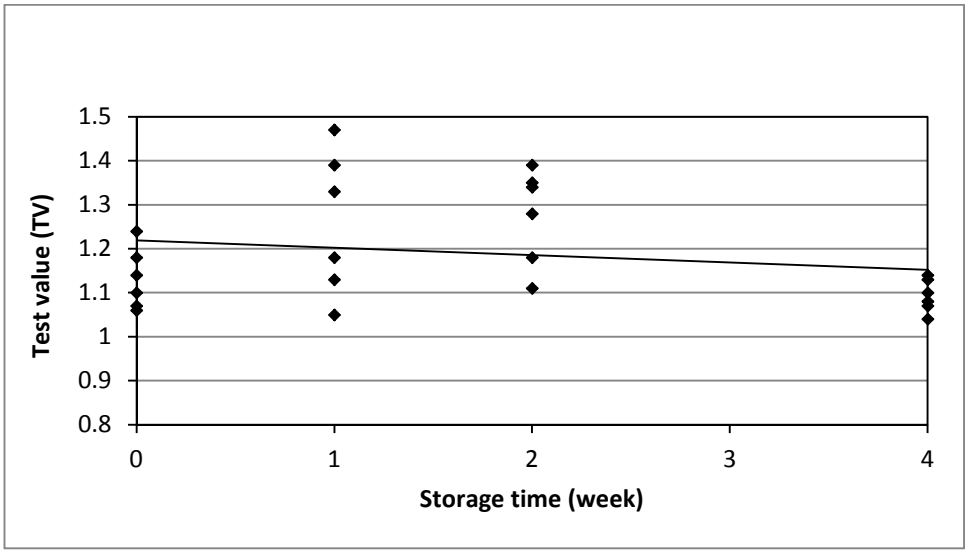

Figure B2. Results of the short-term stability measurements (IRMM-359b, 60 ºC). The obtained results per individual time point and the respective regression line are shown.

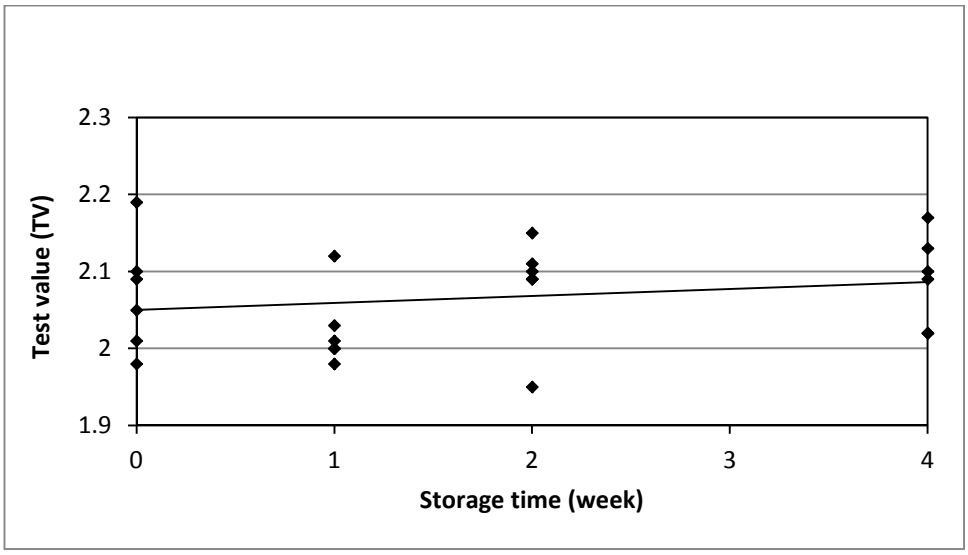

Figure B3. Results of the short-term stability measurements (IRMM-359c, 18 ºC). The obtained results per individual time point and the respective regression line are shown.

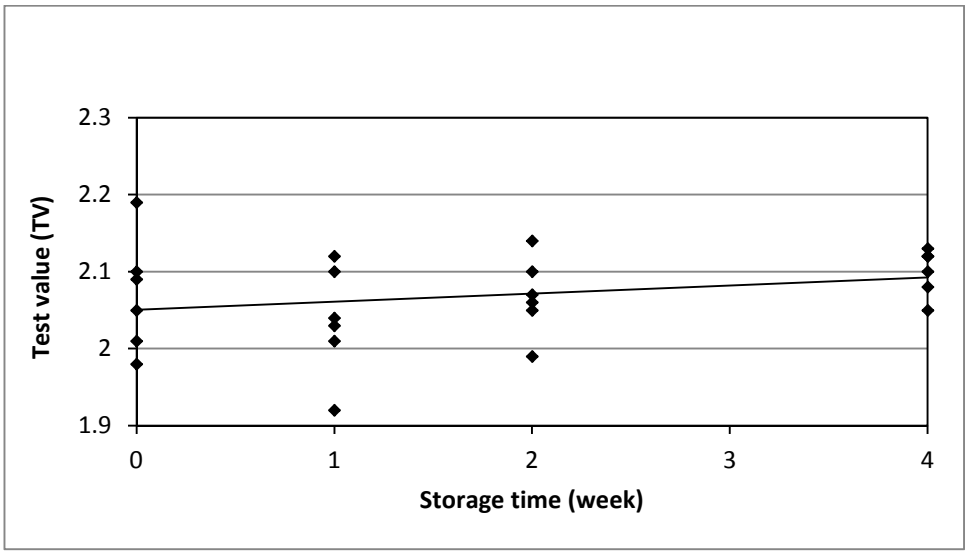

Figure B4. Results of the short-term stability measurements (IRMM-359c, 60 ºC). The obtained results per individual time point and the respective regression line are shown.

Annex C. Long-term stability data

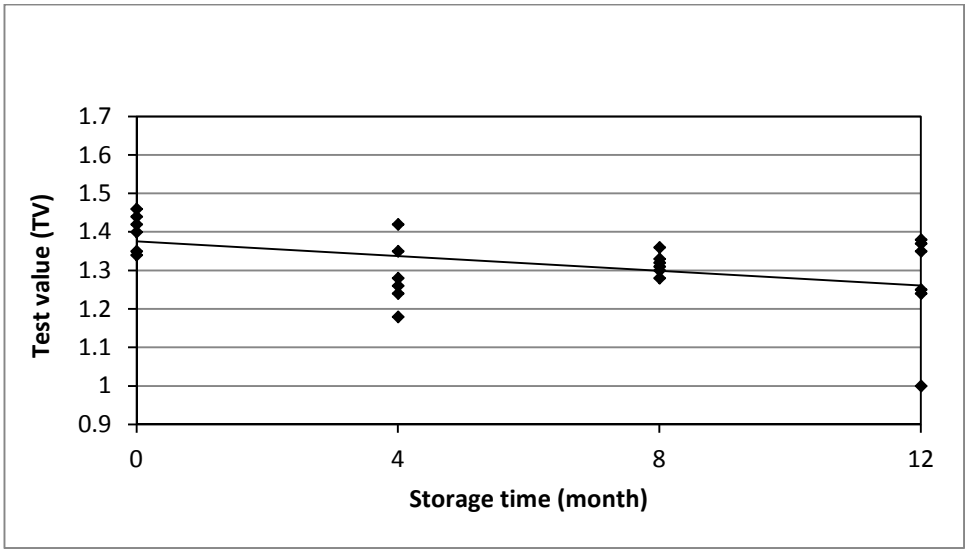

Figure C1. Results of the one year stability measurements (IRMM-359b, 4 ºC). The obtained results per individual time point and the respective regression line are shown.

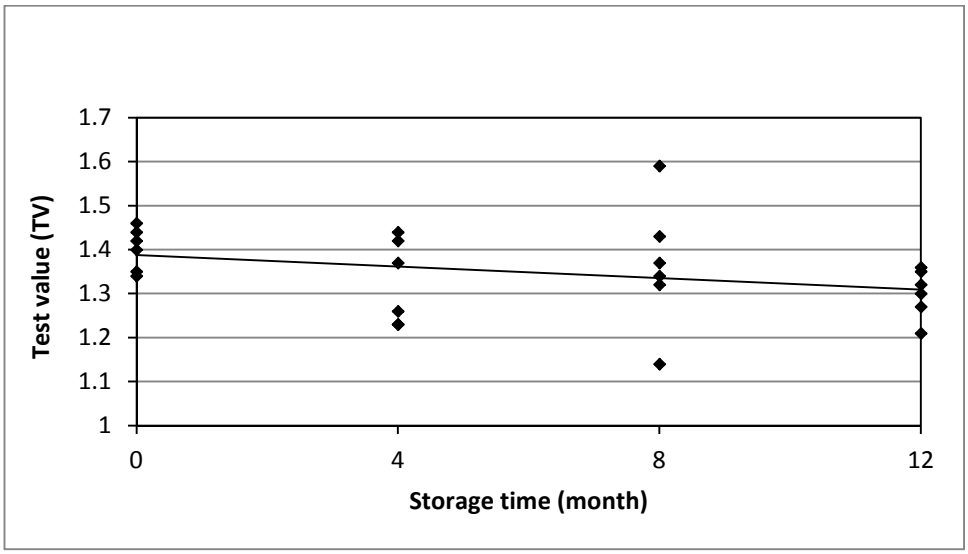

Figure C2. Results of the one year stability measurements (IRMM-359b, -20 ºC). The obtained results per individual time point and the respective regression line are shown.

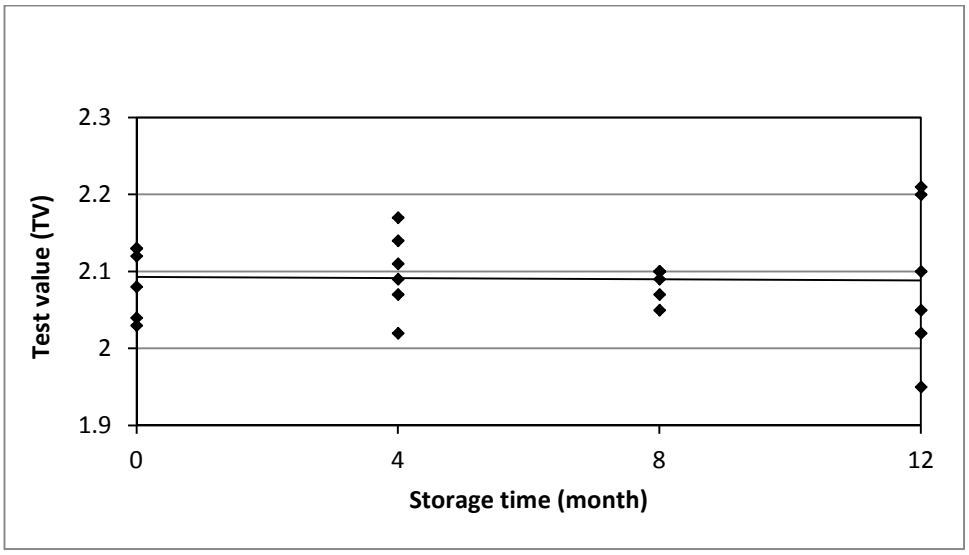

Figure C3. Results of the one year stability measurements (IRMM-359c, 4 ºC). The obtained results per individual time point and the respective regression line are shown.

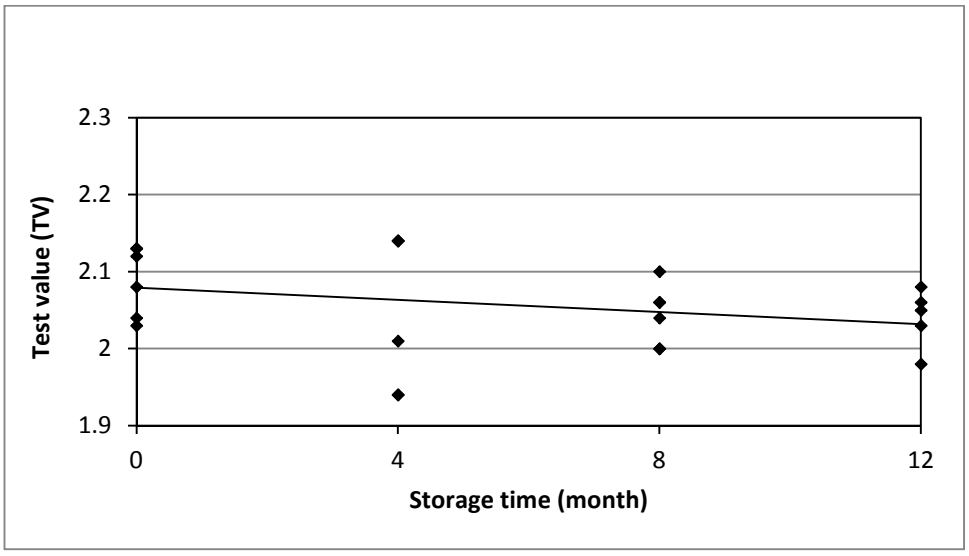

Figure C4. Results of the one year stability measurements (IRMM-359c, -20 ºC). The obtained results per individual time point and the respective regression line are shown.

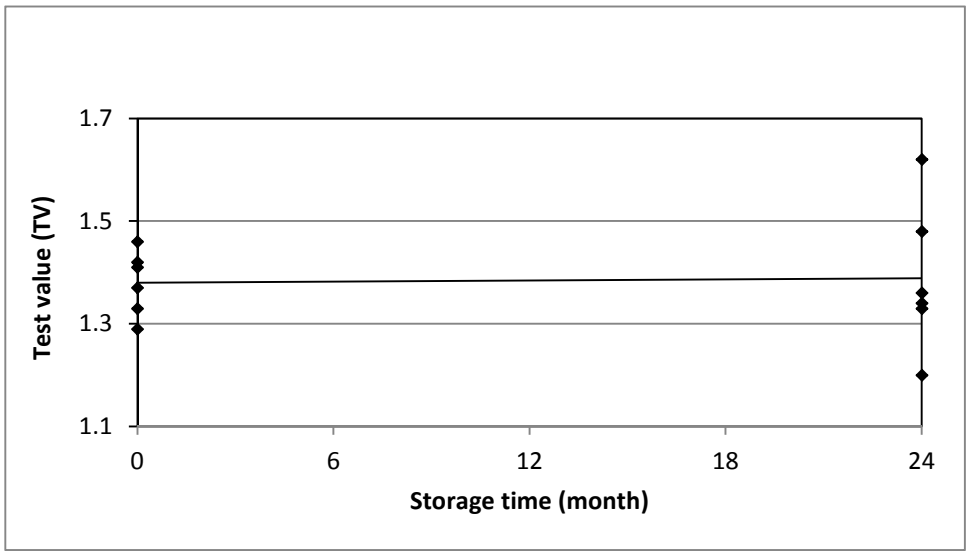

Figure C5. Results of the two year stability measurements (IRMM-359b, 4 ºC). The obtained results per individual time point and the respective regression line are shown.

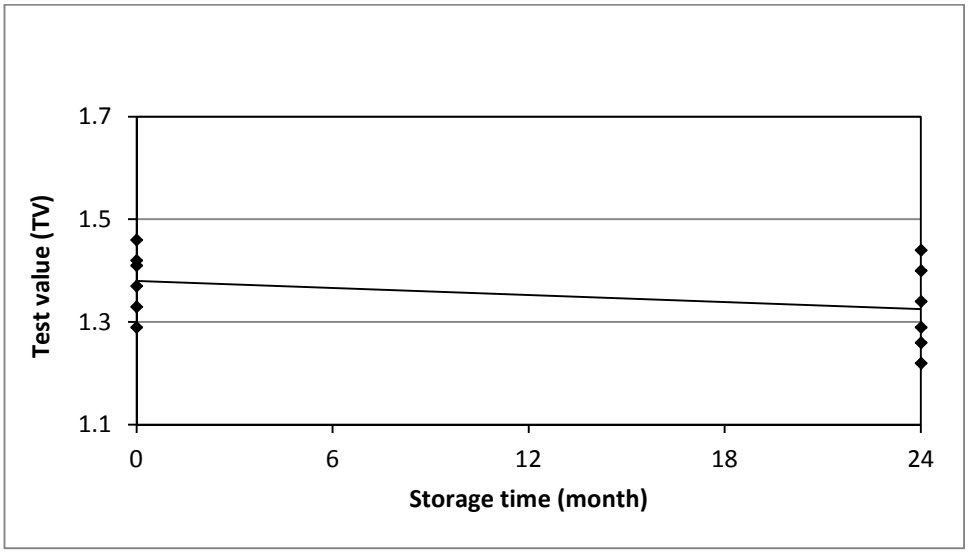

Figure C6. Results of the two year stability measurements (IRMM-359b, -20 ºC). The obtained results per individual time point and the respective regression line are shown.

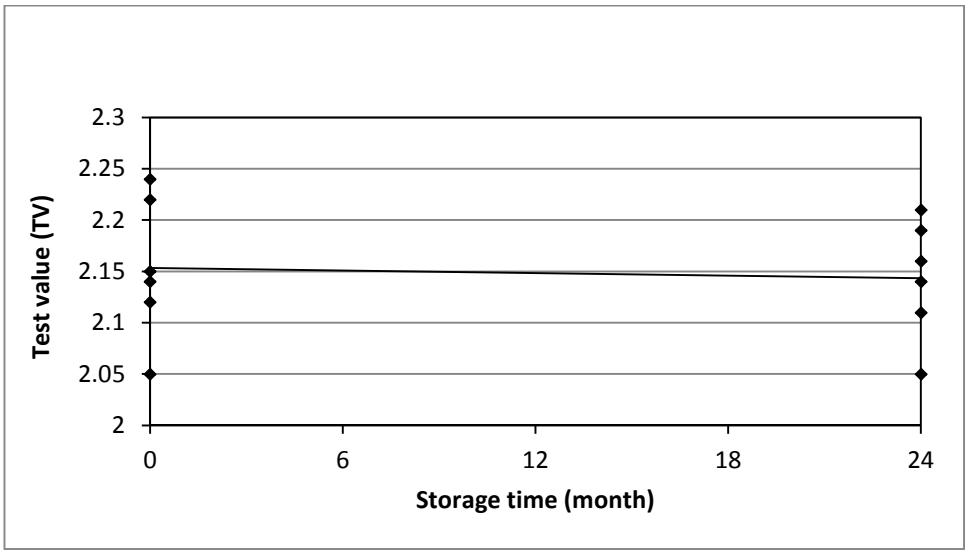

Figure C7. Results of the two year stability measurements (IRMM-359c, 4 ºC). The obtained results per individual time point and the respective regression line are shown.

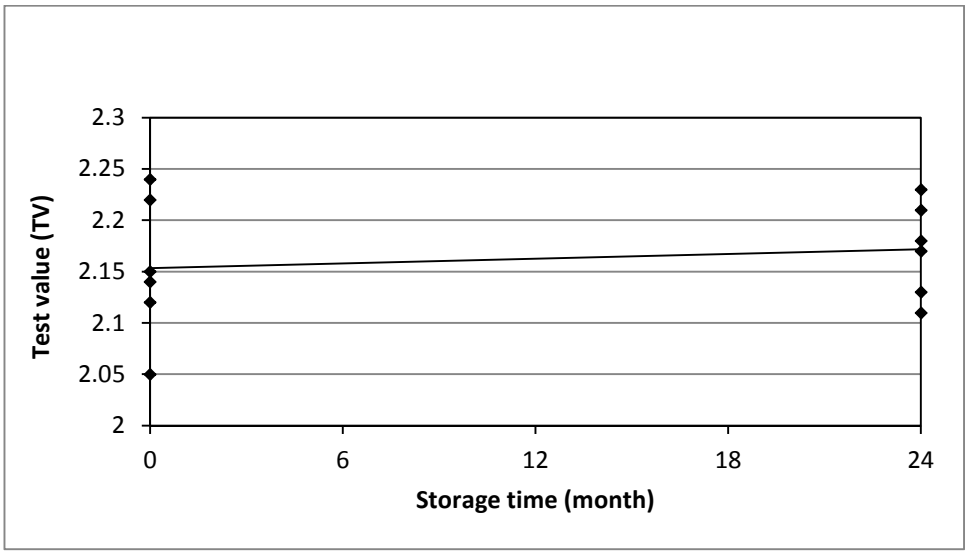

Figure C8. Results of the two year stability measurements (IRMM-359c, -20 ºC). The obtained results per individual time point and the respective regression line are shown.

Annex D. Characterisation data

Table D1. Results of the characterisation measurements for IRMM-359a using the ESM/Ridascreen [4]. The assay is not calibrated in a quantitative manner; the result (expressed in AU) is compared with the threshold of the assay (fixed value of 0.15 AU plus lab-dependent component stemming from repeat measurements of the negative control) to decide whether a sample is judged negative (SEA in the sample < threshold) or positive (SEA in sample ≥ threshold).

Lab code	Day1/1	Day1/2	Day1/3	Day2/1	Day 2/2	Day 2/3	Day 3/1	Day 3/2	Day3/3
A	0.025	0.014	0.025	0.064	0.022	0.034	0.189	0.170	0.148
B	*0.060*	*0.066*	*0.061*	*0.062*	0.069	0.074	0.043	0.036	0.035
C	*0.015*	*0.012*	*0.017*	*0.046*	*0.059*	*0.058*	0.087	*0.100*	*0.068*
D	0.040	0.046	0.044	0.090	0.105	0.066	0.022	0.027	0.027
E	0.090	0.089	0.089	0.069	0.088	0.069	0.091	0.085	0.086
F	0.082	0.134	0.126	0.123	0.092	0.089	0.080	0.112	0.107
G	0.077	0.067	0.067	0.094	0.057	*0.072*	*0.071*	0.073	*0.065*

[1]data in italic not considered for evaluation because prescribed protocol was not strictly followed

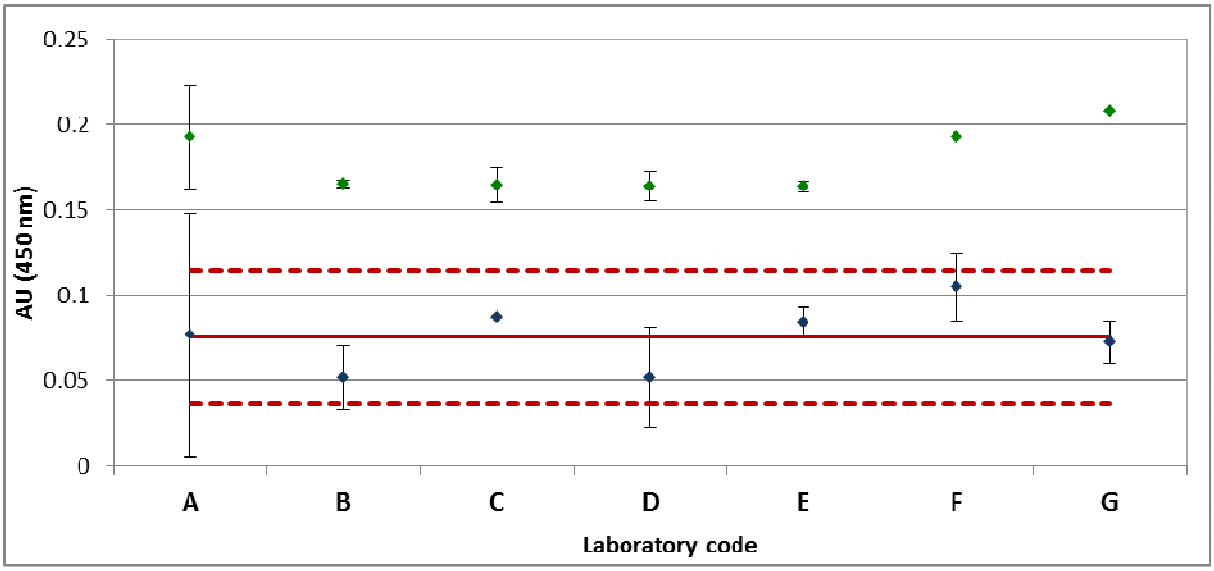

Figure D1. Results of the characterisation study for IRMM-359a, ESM/Ridascreen laboratories. The blue points and the error bars represent the laboratory means and standard deviations. The green points and the error bars represent the laboratory threshold means and standard deviations. The bold red line represents the mean of means, and the dotted red lines represent ± 2 times the standard deviations around the mean of means.

Table D2. Results of the characterisation measurements for IRMM-359b using the ESM/Ridascreen [4]. The assay is not calibrated in a quantitative manner; the result (expressed in AU) is compared with the threshold of the assay (fixed value of 0.15 AU plus lab-dependent component stemming from repeat measurements of the negative control) to decide whether a sample is judged negative (SEA in the sample < threshold) or positive (SEA in sample ≥ threshold).

Lab code	Day1/1	Day1/2	Day1/3	Day2/1	Day 2/2	Day 2/3	Day 3/1	Day 3/2	Day3/3
A	0.367	0.540	0.275	0.541	0.520	0.280	0.651	0.728	0.615
B	*0.610*	*0.798*	*0.760*	0.714	*0.774*	*0.824*	0.726	*0.765*	0.701
C	0.548	0.454	0.473	0.696	0.647	0.548	0.482	0.572	0.670
D	0.847	0.765	0.886	1.111	1.040	1.015	0.717	0.784	0.778
E	0.550	0.434	0.513	0.426	0.436	0.459	0.441	0.489	0.494
F	0.638	0.744	0.628	0.650	0.555	0.791	0.585	0.713	0.717
G	0.360	0.380	0.489	0.438	0.482	0.513	0.409	0.380	0.692

[1]data in italic not considered for evaluation because prescribed protocol was not strictly followed

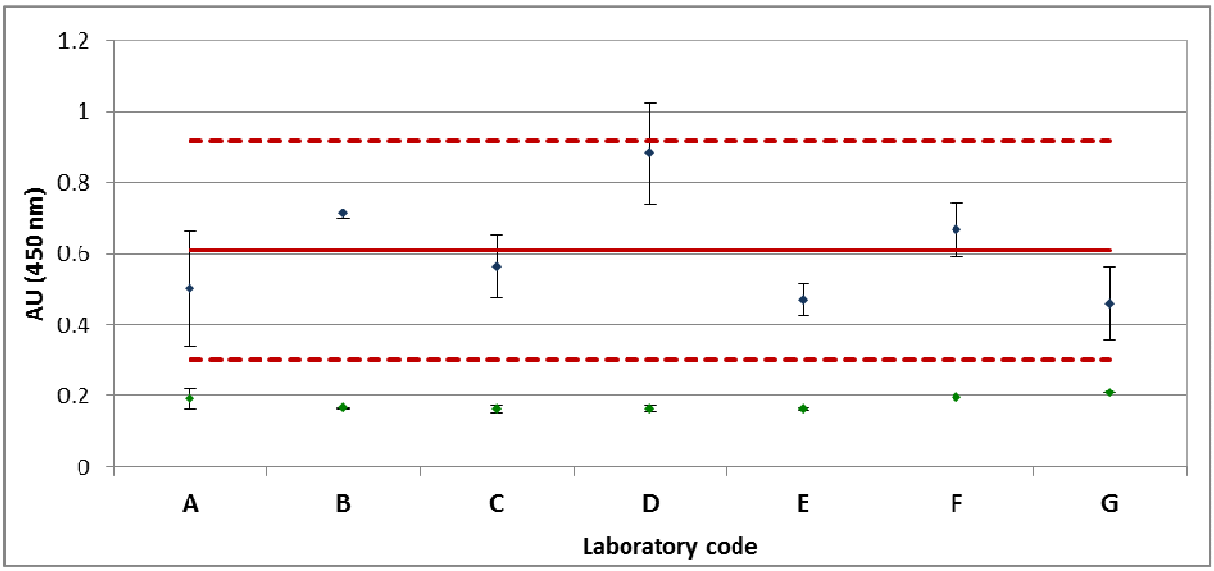

Figure D2. Results of the characterisation study for IRMM-359b, ESM/Ridascreen laboratories. The blue points and the error bars represent the laboratory means and standard deviations. The green points and the error bars represent the laboratory threshold means and standard deviations. The bold red line represents the mean of means, and the dotted red lines represent ± 2 times the standard deviations around the mean of means.

Table D3. Results of the characterisation measurements for IRMM-359c using the ESM/Ridascreen [4]. The assay is not calibrated in a quantitative manner; the result (expressed in AU) is compared with the threshold of the assay (fixed value of 0.15 AU plus lab-dependent component stemming from repeat measurements of the negative control) to decide whether a sample is judged negative (SEA in the sample < threshold) or positive (SEA in sample ≥ threshold).

Lab code	Day1/1	Day1/2	Day1/3	Day2/1	Day 2/2	Day 2/3	Day 3/1	Day 3/2	Day3/3
A	0.767	1.074	0.969	0.757	1.06	0.975	1.391	1.229	1.73
B	1.917	1.835	1.793	*1.643*	1.473	1.539	1.353	1.329	1.278
C	1.911	1.224	1.279	1.320	1.442	1.620	1.722	1.564	1.673
D	1.790	1.844	1.741	2.312	2.288	2.088	1.554	1.789	1.565
E	0.989	1.142	0.913	1.108	1.076	1.162	1.106	1.210	1.017
F	1.718	1.317	1.355	1.615	1.597	1.520	1.345	1.478	1.629
G	*0.817*	0.968	0.953	0.902	0.995	0.645	0.741	0.882	0.45

[1]data in italic not considered for evaluation because prescribed protocol was not strictly followed

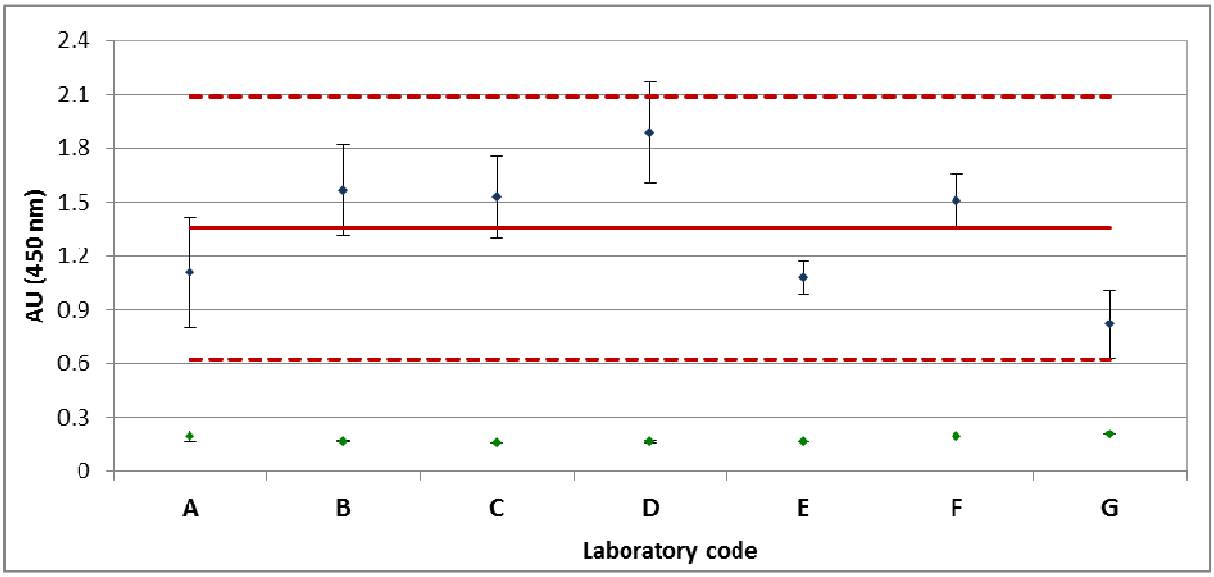

Figure D3. Results of the characterisation study for IRMM-359c, ESM/Ridascreen laboratories. The blue points and the error bars represent the laboratory means and standard deviations. The green points and the error bars represent the laboratory threshold means and standard deviations. The bold red line represents the mean of means, and the dotted red lines represent ± 2 times the standard deviations around the mean of means.

Table D4. Results of the characterisation measurements for IRMM-359a using the ESM/VIDAS [4]. The assay is not calibrated in a quantitative manner; the result (TV) is compared with the threshold of the assay (fixed value of 0.13) in order to decide whether a sample is judged negative (SEA in the sample < 0.13) or positive (SEA in sample ≥ 0.13).

Lab code	Day1/1	Day1/2	Day1/3	Day2/1	Day 2/2	Day 2/3	Day 3/1	Day 3/2	Day3/3
H	0.01	0.01	0.01	0.01	0.01	0	0.01	0.01	0.01
I	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0	0.01
J	0.01	0.01	0.01	0	0.01	0.01	0.01	0.01	0.05
K	*0*	*0*	*0*	0	*0*	*0*	*0*	*0*	*0*
L	0.01	0.02	0.01	0.02	0.02	0.02	0.01	0.01	0.01
M	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
N	0.01	0.02	0.01	0	0	0	0	0	0
O	0.01	0.01	0.01	0.02	0.02	0.02	0.04	0.01	0.02

[1]data in italic not considered for evaluation because prescribed protocol was not strictly followed

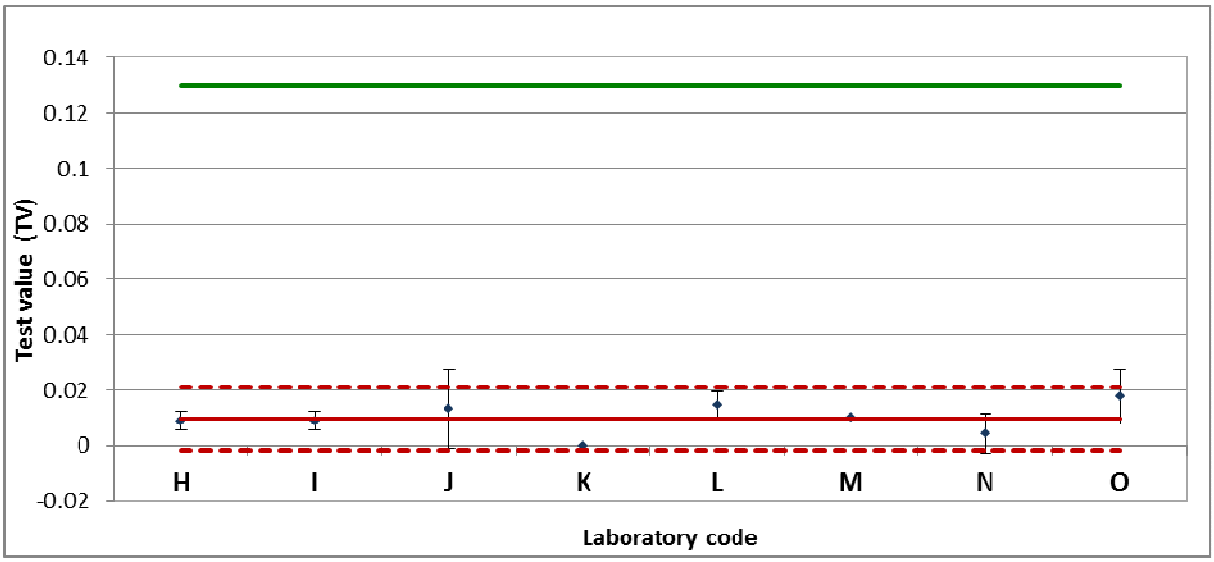

Figure D4. Results of the characterisation study for IRMM-359a, ESM/VIDAS laboratories. The blue points and the error bars represent the laboratory means and standard deviations. The bold green line represents the detection threshold of the assay. The bold red line represents the mean of means, and the dotted red lines represent ± 2 times the standard deviations around the mean of means.

Table D5. Results of the characterisation measurements for IRMM-359b using the ESM/VIDAS [4]. The assay is not calibrated in a quantitative manner; the result (TV) is compared with the threshold of the assay (fixed value of 0.13) in order to decide whether a sample is judged negative (SEA in the sample < LOD of method) or positive (SEA in sample above threshold).

Lab code	Day1/1	Day1/2	Day1/3	Day2/1	Day 2/2	Day 2/3	Day 3/1	Day 3/2	Day3/3
H	1.53	1.37	1.36	1.26	1.24	1.25	1.46	1.29	1.42
I	1.18	1.05	1.00	1.13	1.23	1.32	1.21	1.20	1.11
J	1.21	1.10	1.11	1.32	1.17	1.37	1.28	1.17	1.30
K	*0.97*	0.97	*0.92*	0.54	*0.52*	*0.40*	*0.54*	*0.64*	*0.72*
L	1.39	1.47	1.51	1.19	1.39	1.38	1.27	1.29	1.39
M	0.97	0.77	0.63	0.94	0.77	0.47	0.90	1.07	0.84
N	1.07	1.00	1.21	1.22	1.36	1.40	0.89	1.18	1.25
O	1.25	1.53	1.22	1.24	1.35	0.58	1.21	1.35	1.30

[1]data in italic not considered for evaluation because prescribed protocol was not strictly followed

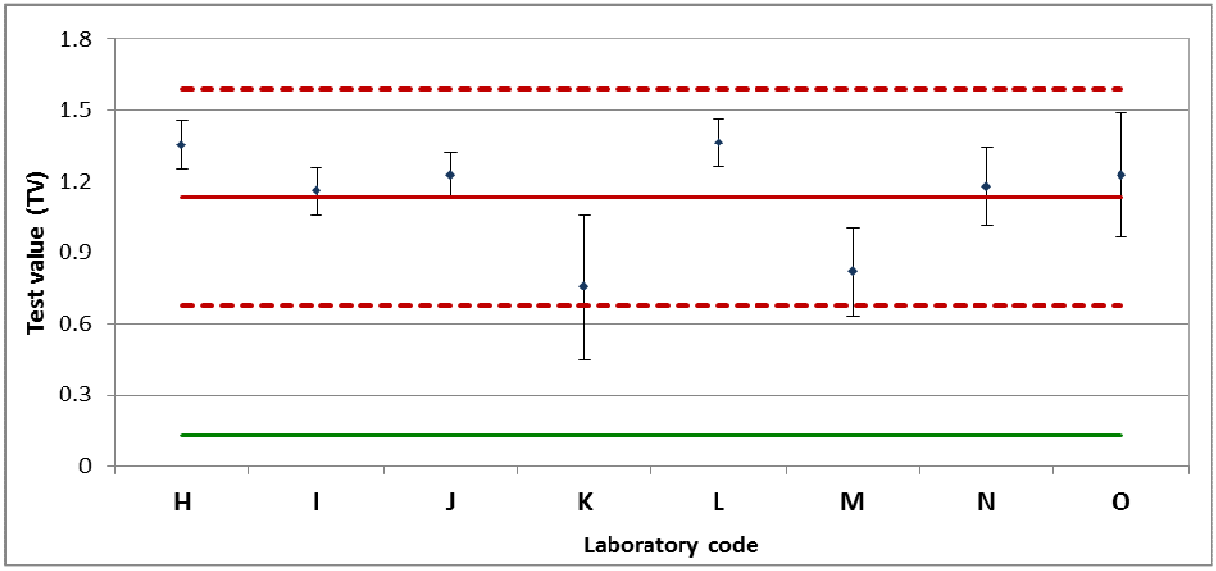

Figure D5. Results of the characterisation study for IRMM-359b, ESM/VIDAS laboratories. The blue points and the error bars represent the laboratory means and standard deviations. The bold green line represents the detection threshold of the assay. The bold red line represents the mean of means, and the dotted red lines represent ± 2 times the standard deviations around the mean of means.

Table D6. Results of the characterisation measurements for IRMM-359c using the ESM/VIDAS [4]. The assay is not calibrated in a quantitative manner; the result (TV) is compared with the threshold of the assay (fixed value of 0.13) in order to decide whether a sample is judged negative (SEA in the sample < 0.13) or positive (SEA in sample above threshold).

Lab code	Day1/1	Day1/2	Day1/3	Day2/1	Day 2/2	Day 2/3	Day 3/1	Day 3/2	Day3/3
H	2.03	1.96	2.13	2.09	2.03	2.12	2.13	2.10	2.17
I	2.08	1.86	2.18	2.01	2.07	2.1	2.03	2.02	2.29
J	1.92	1.73	*2.00*	1.99	1.98	2.00	2.07	2.16	2.12
K	1.80	1.73	*1.71*	*1.42*	*1.40*	*1.37*	*1.50*	*1.54*	*1.10*
L	2.02	2.21	2.23	1.97	2.06	2.20	2.12	2.09	2.20
M	1.10	1.42	1.13	1.39	1.66	1.30	1.64	1.72	1.35
N	1.92	1.91	2.16	2.21	2.29	2.21	2.13	1.87	2.00
O	2.2	2.18	2.30	2.11	2.16	2.11	2.21	2.17	2.42

[1]data in italic not considered for evaluation because prescribed protocol was not strictly followed

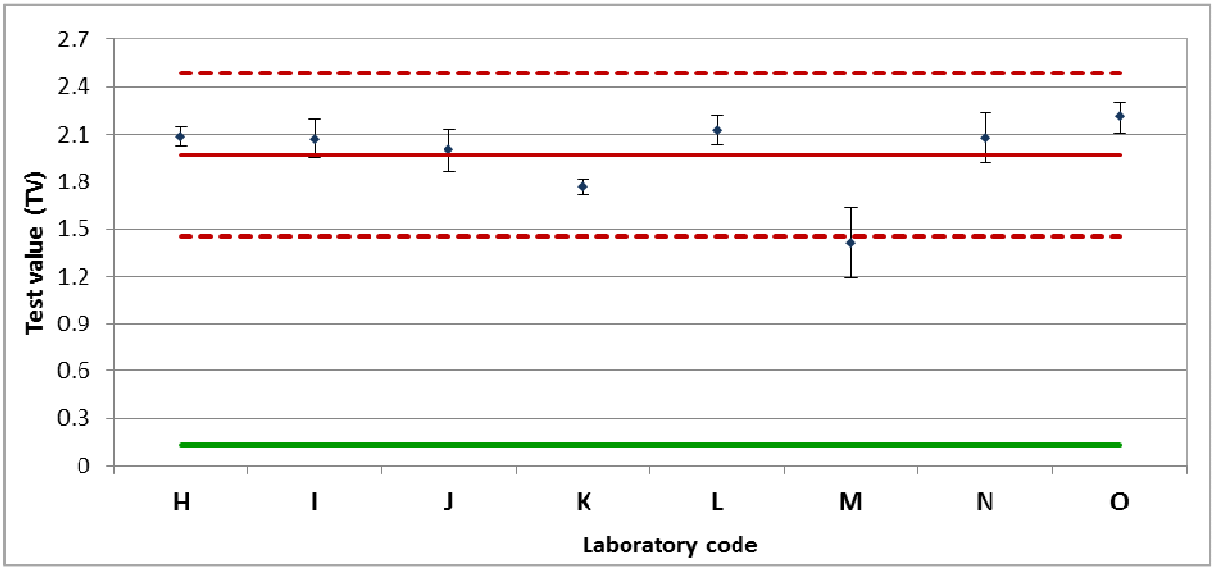

Figure D6. Results of the characterisation study for IRMM-359c, ESM/VIDAS laboratories. The blue points and the error bars represent the laboratory means and standard deviations. The bold green line represents the detection threshold of the assay. The bold red line represents the mean of means, and the dotted red lines represent ± 2 times the standard deviations around the mean of means.

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