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REPORT

The preparation and characterisation of three solutions of plasmid DNA containing a ruminant-specific fragment with defined copy number concentrations

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Summary

This report describes the processing and characterisation of a set of plasmid solutions, IRMM-AD482a, b and c. The material was produced following ISO Guide 34:2009 [1].
A DNA fragment specific for the identification of ruminant meat was cloned into a pUC18 vector to construct the pIRMM-0103 plasmid. The nucleic acid sequence identity of the pIRMM-0103 plasmid was determined by dye terminator cycle sequencing of the entire plasmid. The plasmid was put into a solution and its concentration was measured by ultraviolet (UV) spectrophotometry. Afterwards this solution was diluted gravimetrically to obtain three different plasmid concentration levels. The three concentration levels were verified by digital quantitative polymerase chain reaction (dPCR).

Between-unit homogeneity and stability during dispatch were assessed in accordance with ISO Guide 35:2006 [2].

The materials are intended for the determination of a cut-off value to discriminate positive samples (containing the ruminant target sequence) from negative samples by quantitative PCR. As any reference material (RM), the materials can also be used for control charts or precision studies. The RM is available as a set of three plastic vials containing at least 1 mL of plasmid solution. The minimum amount of sample to be used is 4 µL.

The following indicative values were assigned:

	Copy number concentration of the plasmid [1)]
	indicative value [cp/µL]
IRMM-AD482a	128
IRMM-AD482b	32
IRMM-AD482c	8

[1)] The copy number concentration of the plasmid has been determined by UV spectrophotometry and verified by dPCR using conditions as described in Annex A.

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Glossary

ANOVA	Analysis of variance
b	Slope in the equation of linear regression $y = a + bx$
cp	Number of copies
Cq	Quantification threshold
CI	Confidence interval
CRM	Certified reference material
DNA	Deoxyribonucleic acid
EC	European Commission
EDTA	Ethylenediaminetetraacetic acid
EU	European Union
EURL-AP	European Union Reference Laboratory for Animal Proteins in Feedingstuffs
IRMM	Institute for Reference Materials and Measurements of the JRC
ISO	International Organization for Standardization
JRC	Joint Research Centre
LB	Luria-Broth medium
M	Molar mass
$MS_{between}$	Mean of squares between-unit from an ANOVA
MS_{within}	Mean of squares within-unit from an ANOVA
n	Number of replicates per unit
N	Number of samples (=units) analysed
n.a.	Not applicable
n.c.	Not calculated
PAPs	Processed animal proteins
qPCR	Quantitative polymerase chain reaction
dPCR	Digital qPCR
ddPCR	Digital droplet qPCR
rel	Index denoting relative figures (uncertainties etc.)
RM	Reference material
RSD	Relative standard deviation
s	Standard deviation
s_{bb}	Between-unit standard deviation; an additional index "rel" is added when appropriate
$s_{between}$	Standard deviation between groups as obtained from ANOVA; an additional index "rel" is added as appropriate

s_{meas}	Standard deviation of measurement data; an additional index "rel" is added as appropriate
s_{within}	Standard deviation within groups as obtained from ANOVA; an additional index "rel" is added as appropriate
s_{wb}	Within-unit standard deviation
T	Temperature
t	Time
TaqMan®	*Thermus aquaticus* (*Taq*) DNA polymerase-based technology for fluorescent signal generation during in qPCR
TE	Buffer containing TRIS and EDTA
TRIS	Tris(hydroxymethyl)aminomethane
u	Standard uncertainty
u^*_{bb}	Standard uncertainty related to a maximum between-unit inhomogeneity that could be hidden by method repeatability; an additional index "rel" is added as appropriate
u_{bb}	Standard uncertainty related to a possible between-unit inhomogeneity; 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
UV	Ultraviolet
V	Volume
α	Significance level
$\nu_{s,meas}$	Degrees of freedom for the determination of the standard deviation s_{meas}
$\nu_{MSwithin}$	Degrees of freedom of MS_{within}

1 Introduction

1.1 Background

Since the outbreak of bovine spongiform encephalopathy (BSE), the use of processed animal proteins (PAPs) including meat and bone meal as feed ingredients for farmed animals is regulated within the European Union through several regulations (Regulation (EC) 999/2001, Regulation (EC) 1774/2002 and Regulation (EC) 1234/2003) [3-5]. Regulation (EC) No 152/2009 of 27 January 2009 has laid down the methods of sampling and analysis for the official control of feed [6]. The new Regulation (EU) No 51/2013 of 16 January 2013 includes new methods for the detection of animal constituents based on polymerase chain reaction (PCR) [7]. PCR methods have been designed to identify unauthorised proteins from ruminant origin. The PCR methods are targeting remaining intact DNA fragments from ruminant origin that still can be detected in heavily processed feed samples.
Such a PCR method has been recently validated by the EU Reference Laboratory for animal proteins (EURL-AP) in feeding stuffs. Calibrants are needed for the correct implementation of this PCR test used as an official control for detecting the presence of unauthorised ruminant material in feed. Control laboratories must indeed be able to distinguish accurately positive from negative results. This discrimination is made by determining a threshold at which a PCR signal is considered as being negative. This threshold (or cut-off value) may vary from one laboratory to another as quantification threshold (Cq) values obtained by quantitative PCR (qPCR) are influenced by a large number of factors including the composition of the mastermix and the instrument used [8]. Therefore each control laboratory needs to determine its individual threshold value and to verify this value on a regular basis according to its quality system. To determine a cut-off value, three plasmid solutions (each containing a precise concentration of the specific ruminant target) are tested by the official control laboratories in parallel with their real samples.

The Joint Research Centre - Institute for Reference Materials and Measurements (JRC-IRMM) has been asked to produce a calibration kit containing three plasmid solutions. The solutions are characterised for their DNA copy number concentration and allow control laboratories to determine their cut-off values and to report their results in line with European Commission Regulation No 51/2013 [7].

1.2 Choice of the material

There are several requirements for a material to be used as a calibrant for qPCR, in addition to requirements for homogeneity, stability and metrological traceability of assigned values.

The calibrants are used to determine a cut-off value to discriminate positive from negative samples tested for the presence of a ruminant target.

To determine a cut-off value, a calibration curve is made using three plasmid solutions each containing a defined copy number concentration of a specific ruminant target. The plasmid that contains the target sequence for the ruminant assay has been constructed by the EURL-AP. The ruminant target is an extremely abundant nuclear DNA fragment of 85 to 86 base pairs that can be amplified using two specific primers. The three calibrants will be certified for their absolute copy number concentration at a later stage.

Until then, was decided to produce a first set of three plasmid solutions with indicative copy number concentrations of 128 cp/µL, 32 cp/µL and 8 cp/µL. These concentration levels are

requested by the EURL-AP to allow control laboratories to calculate the cut-off value of their ruminant assay [9].

1.3 Design of the project

A purified plasmid DNA solution was measured by UV spectrophotometry. Appropriate concentration levels for the 3 solutions at nominal values of 128 cp/µL, 32 cp/µL and 8 cp/µL were obtained by gravimetric dilution of this stock solution. Those concentrations have been verified by digital qPCR (dPCR). A short term-stability study, a freeze-thaw cycle study and a homogeneity study have been carried out.

2 Participants

2.1 Raw material provider

EURL-AP, Gembloux, BE

2.2 Project management and evaluation, processing, homogeneity and stability studies

European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE
(accredited to ISO Guide 34 for production of certified reference materials, BELAC No. 268-RM)

3 Material processing and process control

3.1 Origin and purity of the starting material

The plasmid was received from the EURL-AP as a bacterial culture in Luria broth (LB) medium supplemented with glycerol. The plasmid was extracted from an isolated colony, purified on a silica column (Qiaquick Gel extraction kit, Qiagen, Venlo, The Netherlands) and suspended in TE low buffer (1 mM Tris, 0.01 mM EDTA, pH 8).

3.1.1 Identity confirmation

The identity of the plasmid pIRMM-0103 was confirmed by restriction enzyme digestion followed by agarose gel electrophoresis (Figure 1). The plasmid was either undigested (lane 5) or digested with *EcoR*V and *BamH*I (resulting in a 147 bp and a 2713 bp fragment [lane 2]), *Hind*III and *BamH*I (resulting in a 204 bp and a 2656 bp fragment [lane 3]) and *Hind*III (resulting in a 2684 bp fragment [lane 4]) and these DNAs were visualised in a 1 % m/m agarose gel by UV after ethidium bromide staining. DNAs with their respective sizes were observed.

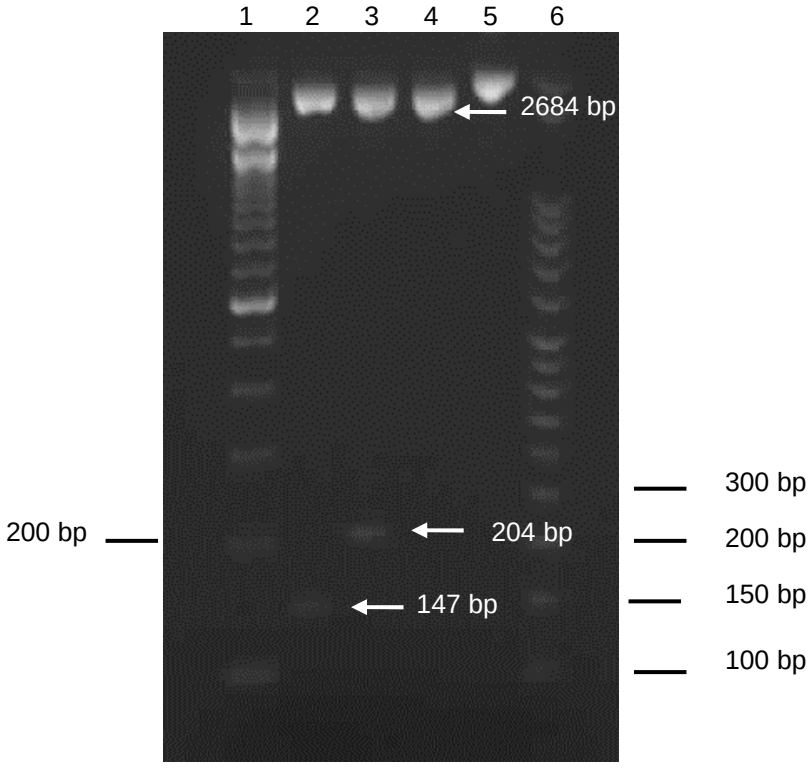

Figure 1: Restriction enzyme digestion analysis of pIRMM-0103. Lane 1: 100 bp DNA ladder (cat 15628-019, Invitrogen, Life Technologies Europe, Gent, BE), Lane 2: pIRMM-0103 digested with *EcoRV and BamH*I (expected fragments: 147 bp and 2713 bp); Lane 3: pIRMM-0103 digested with *Hind*III and *BamH*I (expected fragments: 204 bp and 2656 bp); Lane 4: pIRMM-0103 digested with *Hind*III (plasmid linearisation expected); Lane 5: undigested pIRMM-0103; Lane 6: DNA Step ladder 50 bp ladder (S7025-Sigma Aldrich BVBA, Diegem, BE). The size of DNA fragments reported above 1000 bp are theoretical values.

3.1.2 Purity of the plasmid extract

In theory three different types of contaminants could be present in the plasmid extract: a) intermediate plasmids used for the assembly of pIRMM-0103, b) nucleic acids originating from host bacterial cells and c) non-nucleic acid contaminants.

During the cloning process the bacterial cells could have been transfected with different populations of plasmids: pIRMM-0103 and one or more of the intermediate plasmids used for the assembly of pIRMM-0103. The synthetic plasmids used in the cloning strategy (pUC18 and pCR 2.1) have the same origin of replication (*ori*V from ColE1 plasmid) which allows them to replicate independently of the host chromosome. A bacterial cell, however, cannot replicate different plasmids with the same mechanism of replication. As a consequence only one plasmid will remain present in a bacterial clone while others are lost during cell division. As the plasmid production was started from a single colony, only one type of plasmid can be present in the plasmid extract [10]. To ensure that the plasmid production started from a single colony, one additional plating step was included. A single colony containing the pIRMM-0103 was plated on a LB plate and from this plate one isolated colony was picked for processing.

After enzymatic digestion with *Hind*III and purification with the Qiaquick® Gel extraction kit, the purified plasmid solution was analysed by gel electrophoresis. As there was no smear and/or RNA bands observed, it can be reasonably concluded that the plasmid preparation was not contaminated with large amounts of genomic DNA or RNA molecules from host bacterial cells. However, traces of genomic DNA or RNA molecules cannot be detected by gel electrophoresis.

The linearised plasmid was investigated by spectrophotometry to detect the presence of non-nucleic acid contaminants like proteins, or chemical residues from nucleic acid extraction like phenol and guanidine. The UV absorbance of the plasmid in solution was measured at 230 nm (A_{230}), 260 nm (A_{260}) and 280 nm (A_{280}). Taking into account the generally accepted mean extinction coefficient for double-stranded DNA at 260 nm and 280 nm, pure nucleic acid samples are expected to have an A_{260}/A_{280} ratio of approximately 1.8 and an A_{260}/A_{230} ratio of approximately 2.0 or higher [11]. The A_{260}/A_{280} and A_{260}/A_{230} ratios measured were 1.86 and 1.97 respectively, indicating a sufficient DNA purity; however, such values do not exclude traces of contaminating proteins or chemical residues from nucleic acid extraction.

Contamination of the stock solution of plasmid pIRMM-0103 with traces of nucleic acids for the host bacterial cell proteins may affect the DNA mass concentration measured by spectrophotometry and fluorometry. This may lead to an overestimation of the copy number concentration of the plasmid in solution. However, such traces do not affect the dPCR measurements as the used primers and probes are highly specific for the targeted sequences within a dPCR and do not hybridise to other DNA fragments that could be present in the final plasmid preparation. Traces of chemical residuals from nucleic acid extraction like phenol and guanidine might, however, inhibit the PCR reaction and lead to an underestimation of the copy number concentration of a highly concentrated plasmid solution. Inconsistencies between the estimations of the copy number concentration based on spectrophotometry and dPCR can therefore be used as an indication for the presence of contaminants in the plasmid solution.

Based on DNA mass concentration, the copy number concentration of the plasmid solution was estimated, applying the following equation.

$$copy\ number\ concentration = \frac{c_{DNA} \cdot N_A}{M_{plasmid}} \qquad \textbf{Equation 1}$$

c_{DNA} DNA mass concentration

N_A Avogadro constant

$M_{plasmid}$ molar mass of the pIRMM0103 plasmid

Calculation of the molar mass of the pIRMM0103 plasmid was based on the DNA sequence of the plasmid and the molar mass of each nucleotide monophosphate [12].

Table 1 shows the results obtained with these two different techniques. The measurement results and their standard deviations obtained for the copy number concentration are overlapping.

Table 1: The DNA mass concentrations (c_{DNA}) and the estimated copy number concentrations (with their standard deviation [s]) obtained for the stock solution of linearised plasmid pIRMM-0103 using two different methods

Method	Number of replicates	Mean $c_{DNA} \pm s$ [ng/µL]	Copy number concentration $\pm s$ [10^{10}cp/µL]
UV spectrophotometry	3	53.62 ± 0.41 (measured)	1.83 ± 0.14 (estimated)
dPCR	9	54.80 ± 2.31 (estimated)	1.88 ± 0.08 (measured)

Based on all these observations and knowing that the plasmid stock solution is diluted by at least a factor of 140 million to generate the final plasmid solutions, it can be reasonably concluded that there are no major contaminations present in the plasmid solution that might affect the amplification of the target DNA in qPCR.

3.2 Processing

The plasmid concentration was estimated by UV spectrophotometry (resulting in 53.62 ng/µL) and this plasmid stock solution was diluted in TE containing maize genomic DNA.

Maize genomic DNA was isolated and purified from untreated maize seeds. The purity was tested spectrophotometrically and the absence of signals with the ruminant assay was tested by dPCR. The maize genomic DNA was submitted to 3 successive autoclavings to fragmentise the DNA. The fragmentation was verified by gel electrophoresis.

The plasmid solution was diluted gravimetrically in TE containing 12 ng maize DNA/µL to 1280 cp/µL. From this intermediate solution, three independent dilutions were gravimetrically prepared to obtain 1.2 L of solution each with the following indicative concentrations: 128 cp/µL, 32 cp/µL and 8 cp/µL.

From each final solution a volume of approximately 1.1 mL was dispensed semi-automatically in 1000 sterile Axygen pre-labelled low binding vials under sterile conditions. The filling was performed using a liquid dispensing device with cooling elements around the plasmid solution vessel.

Once filled, the vials were closed manually, placed per 100 in boxes that were then sealed and stored at -20 °C. The kits were assembled into a plastic box containing one vial of each concentration level (Figures 2 and 3) and stored back at -20 °C. During this assembly process, the plasmid solutions haven been kept in a frozen stage.

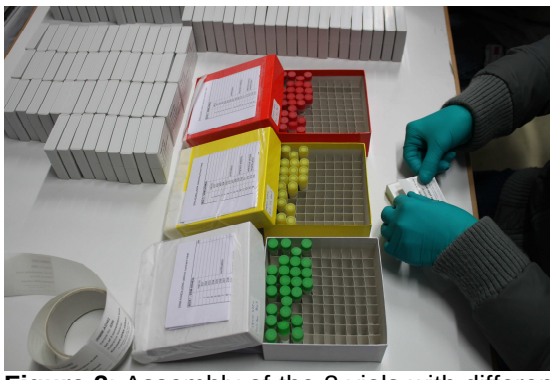

Figure 2: Assembly of the 3 vials with different concentration in each IRMM-AD482 Kit. This work was performed in a cold room at +4°C.

Figure 3: IRMM-AD482 Kit sample N°0265 containing 3 vials of plasmid solutions at indicative concentration levels of 128 cp/µL, 32 cp/µL and 8 cp/µL.

3.3 Process control

Before each dispensing, each indicative final plasmid concentration was verified by dPCR as described in Annex A. The copy number concentration was also verified by droplet dPCR (ddPCR) in a QX100 droplet digital PCR system (Bio-Rad Laboratories N.V., Nazareth Eke, BE) following the manufacturer recommendation and using the SuperMix reagent. The measured copy number concentrations were in close agreement with the dPCR measurements [data not shown].

4 Homogeneity of the copy number concentration of the plasmid

A key requirement for any reference material is the equivalence between the various units. ISO Guide 34 requires RM producers to quantify the between-unit variation. This aspect is covered by between-unit homogeneity studies.

This homogeneity study was planned together with the measurements to control the gravimetric dilutions of the three plasmid solutions. As the measurement results were obtained under repeatability conditions on vials randomly taken from the entire batch and analysed in a randomised order they were as well suited to investigate the homogeneity.

4.1 Between-unit homogeneity

The between-unit homogeneity was evaluated for each concentration level of IRMM AD482.

The number of selected units corresponds to the cubic root of the total number of the produced units (1000). 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 10 groups (with similar number of vials) and one vial was randomly selected from each group. Each selected unit of each concentration level was analysed 5 times independently by dPCR. The measurements of the randomised replicates were performed on 15 digital Array ^{TM} IFC's (Fluidigm). The measurements were performed under repeatability conditions and in a randomised manner to be able to separate a potential analytical drift from a trend in the filling sequence.

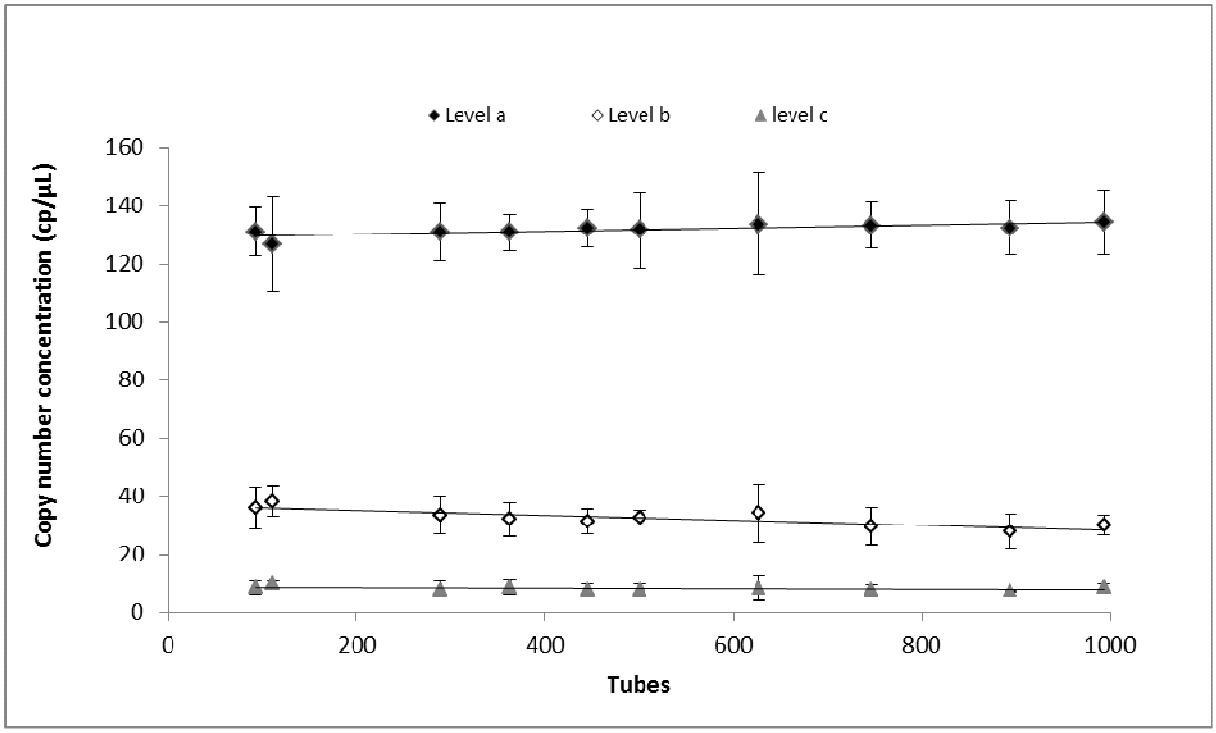

Figure 4: The 3 copy number concentrations measured by dPCR for level a (128 cp/µL), b (32 cp/µL) and c (8 cp/µL) batches. Each point represents the average of 5 measurements, bars indicate the standard deviation at 95 % CI.

Regression analyses were performed to evaluate potential trends in the analytical sequence as well as trends in the filling sequence. No trend in the analytical sequence was visible at a CI of 99 %. A filling trend was detected for IRMM-AD482a, however, no filling trend was observed for IRMM-AD482b and IRMM-AD482c.

The dataset was tested for consistency using Single Grubbs outlier tests on a CI of 99 % on the individual results and the unit means. Some outlying individual results and outlying unit means were detected. Since no technical reason for the outliers could be found, all the data were retained for statistical analysis.

For IRMM-AD482b and IRMM-AD482c, the quantification of between-unit inhomogeneity was accomplished 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 and results for each unit, both following 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. The results of all statistical evaluations are given in Table 2.

Table 2: Results of the statistical evaluation of the between-unit homogeneity study at 99 % CI using dPCR.

Material	Trends (before correction)		Outliers		Distribution	
	Analytical sequence	Filling sequence	Individual results	Unit means	Individual results	Unit means
IRMM-AD482a	no	yes	none	none	unimodal	unimodal
IRMM-AD482b	no	no	none	none	normal	unimodal
IRMM-AD482c	no	no	none	none	normal	normal

One has to bear in mind 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 squares within groups (MS_{within}), resulting in negative arguments under the square root used for the estimation of the between-unit variation in Equation 2, whereas the true variation cannot be lower than zero. In this case, the maximum inhomogeneity that could be hidden by method repeatability ($u^*_{bb)}$ was calculated as described by Linsinger *et al.* [13]. 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 for IRMM-AD482b and for IRMM-AD482c as:

$$s_{wb,rel} = \frac{\sqrt{MS_{within}}}{\bar{y}} \qquad \textbf{Equation 2}$$

$$s_{bb,rel} = \frac{\sqrt{\frac{MS_{between} - MS_{within}}{n}}}{\bar{y}} \qquad \textbf{Equation 3}$$

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

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}

For level a, a more conservative approach was used to estimate the uncertainty related to homogeneity. When a trend in the filling sequence is significant at least at 99 % CI, the uncertainty was assessed using a rectangular distribution between the highest and lowest unit mean. The corrected uncertainty in those cases where there was a significant trend in the filling sequence is given in:

$$u_{rec} = \frac{\left|highest\ mean\ \ result - lowest\ \ mean\ result\right|}{2 \cdot \sqrt{3}\ \cdot \overline{y}}$$ **Equation 5**

The results of the evaluation of the between-unit variation are summarised in Table 3. The resulting values from the above equations were converted into relative uncertainties.

For IRMM-AD482a, the inhomogeneity was quantified as u_{rec} and is still sufficiently small to make the material useful. Therefore, u_{rec} was used as estimate of u_{bb} for level a.

For IRMM-AD482b and IRMM-AD482c, the homogeneity study showed no outlying unit means or trends in the filling sequence. Therefore the between-unit standard deviation (s_{bb}) can be used as estimate of u_{bb}. As u^*_{bb} sets the limits of the study to detect inhomogeneity, the larger value of s_{bb} and u^*_{bb} is adopted as uncertainty contribution to account for potential inhomogeneity.

Table 3: Results of the between-unit homogeneity study

Material	$s_{wb,rel}$ [%]	$s_{bb,rel}$ [%]	$u^*_{bb,rel}$ [%]	$u_{rec,rel}$ [%]	$u_{bb,rel}$ [%]
IRMM-AD482a	n.a.	n.a.	n.a.	10.2	10.2
IRMM-AD482b	18	6.9	3.8	n.a.	6.9
IRMM-AD482c	27.4	n.c.[1)]	5.8	n.a.	5.8

[1)] cannot be calculated as $MS_{between} < MS_{within}$

For the levels b and c, 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}. As u^*_{bb} sets the limits of the study to detect inhomogeneity, the larger value of s_{bb} and u^*_{bb} is adopted as uncertainty contribution to account for potential inhomogeneity.

For level a, the inhomogeneity was quantified as u_{rec} and is still sufficiently small to make the material useful. Therefore, u_{rec} was used as estimate of u_{bb} of level a.

4.2 Within-unit homogeneity and minimum sample intake

The within-unit homogeneity is closely correlated to the minimum sample intake. Due to this correlation, individual aliquots of a material will not contain the same amount of analyte. The minimum sample intake is the minimum amount of sample that is representative for the whole unit and thus can be used in an analysis.

Homogeneity and stability studies were performed using a 4 µL sample intake. This sample intake gives acceptable repeatability, demonstrating that the within-unit inhomogeneity does no longer contribute to analytical variation using this sample intake.

5 Stability

Stability testing is necessary to establish conditions for storage (long-term stability) as well as conditions for dispatch to the customers (short-term stability).

Stability studies were carried out using an isochronous design [14] and under repeatability conditions which greatly improves the sensitivity of the stability tests. In that approach, samples were stored for a certain time at different temperature conditions. Afterwards, the samples were moved to conditions where further degradation can be assumed to be

negligible (reference conditions). At the end of the isochronous storage, the samples were analysed simultaneously under repeatability conditions.

5.1 Short-term stability study

For the short-term stability (STS) study, samples were stored at +4 °C and -20 °C for 0, 1, 2 and 4 weeks (for each testing temperature). The reference temperature was set to -70 °C. Four units per storage time were selected using a random stratified sampling scheme. Each unit was measured once by dPCR. The measurements were performed under repeatability conditions and in a randomised sequence to be able to separate a potential analytical drift from a trend over storage time.

The obtained data were evaluated individually for each temperature. The results were screened for outliers using the single and double Grubbs test. One outlying individual result was found. As no technical reason for the outlier could be found all data were retained for statistical analysis. The results of the statistical evaluation of the short-term stability are summarised in Table 4.

Table 4: **Results of the short-term stability study**

Material	Number of individual outlying results		Significance of the trend at 99% CI	
	+4 ºC	-20 ºC	+4 ºC	-20 ºC
IRMM-AD482a	none	none	yes	no
IRMM-AD482b	none	1 (statistically retained)	no	no
IRMM-AD482c	none	none	no	no

A negative trend was observed for samples of IRMM-AD482a and a slight positive trend but not significant was observed for IRMM-AD482b when kept at +4 °C.

One statistical outlier was detected for IRMM-AD482b at -20 °C, and was retained for the estimation of uncertainty of STS (u_{STS}). None of the trends was statistically significant on a 99 % CI at -20 °C. The material shall be shipped frozen.

A long-term stability for the IRMM-AD482a-c is on-going; the results will be available at a later stage. Due to the urgency of the release of this calibration kit and previous experience on the long-term stability of similar reference materials (ERM-623a-e), we do not expect any instability of this material when stored at -20 °C for longer periods of time.

5.2 Freeze-thaw study

Since the recommended sample intake is 5 µL per concentration level, the establishment of a calibration curve with 3 replicates per concentration level would consume 15 µL of each calibration solution. As there are at least 1000 µL in each vial, at least 60 calibration curves can be prepared. As the storage temperature is -20 °C, repeated use of the RM will require repeated freeze-thaw cycles. In this stability study, the effect of repeated freeze-thaw cycles was investigated.

A similar approach to the isochronous design was carried out. Vials from IRMM-AD482a, IRMM-AD482b and IRMM-AD482c were analysed. For each concentration, two vials were exposed to 0, 8, 16 and 32 freeze-thaw cycles and moved to the reference condition (-70 °C) afterwards.

Each vial was measured 5 times with simplex real time dPCR as described in Annex A. The measurements were done under intermediate precision conditions (Figure 5).

No outlying individual result was found using single Grubbs outlier tests (with 99 % CI). Furthermore, the copy number concentration data were plotted against the number of freeze-thaw cycles and regression line analyses were performed. The slope of the regression lines was then tested for statistical significance (losses or increases due to freezing and thawing). For the test vials of IRMM-AD482b and IRMM-AD482c, the slopes of the regression lines were not significantly different from zero (99 % CI). For the test vials of IRMM-AD482a the slope of the regression line was significantly different from zero (99 % CI). Additionally, a closer investigation of the results for the test vials of ERM-623a showed that there was a significant degradation after 8 cycles.

Although degradation was only observed for one of the three concentration levels, precautions should be taken and the RMs should not be exposed to more than 5 freeze-thaw cycles as a precautionary measure.

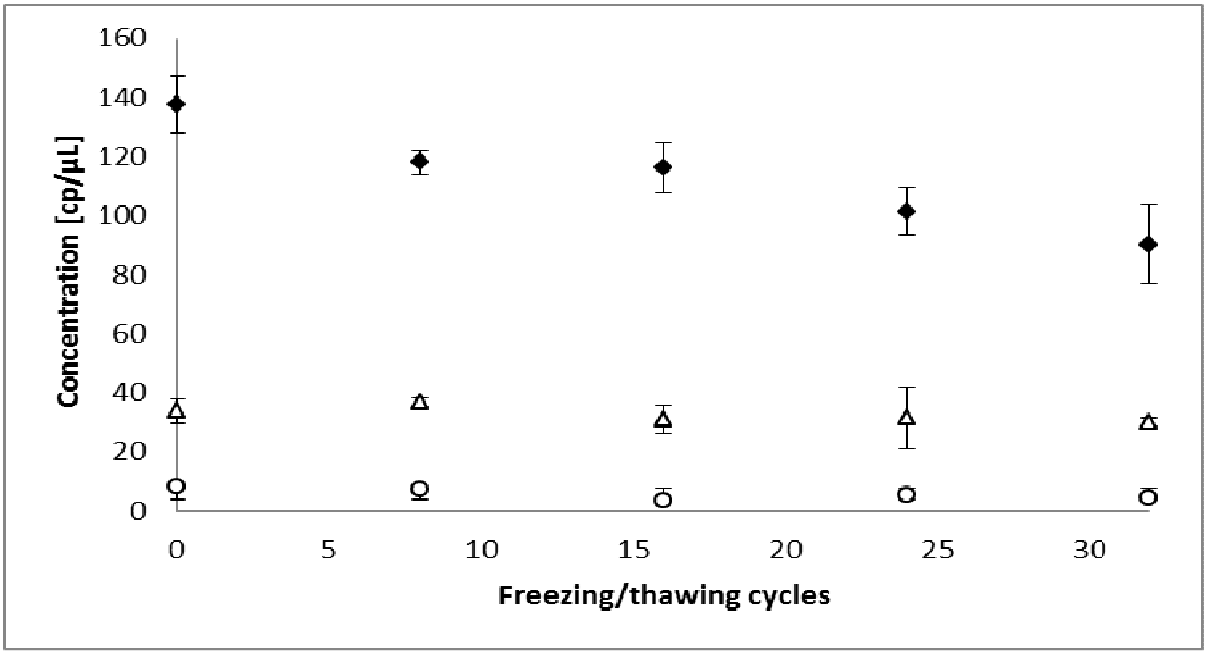

Figure 5: Copy number concentration of the ruminant target for IRMM-AD482a (♦), IRMM-AD482b (Δ) and IRMM-AD482c (o) upon successive freeze-thaw cycles. The bars represent the standard deviation of 2 investigated vials per test freeze-thaw cycle at 95 % CI.

6 Instructions for use

6.1 Safety information

The usual laboratory safety measures apply.

6.2 Storage conditions

The materials shall be stored at -20 °C ± 5 °C prior to use. It is advisable to close the vial with the original screw cap after use. The plasmid containing solution should not be exposed to direct sun light.

Under the condition that major contaminations during handling of opened vials are excluded, the solutions can be used for several experiments. The material should however not pass more than 5 freeze-thaw cycles. It is recommended to aliquot the solutions to reduce the number of freeze-thaw cycles. The material can also be stored at +4°C for 1 week as it was verified that no significant changes to the assigned concentration were observed during that period.

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 opened vials.

6.3 Preparation and use of the material

To make the plasmid solutions ready for use, the contents of the vials have to be thawed completely and mixed gently by inverting the vial several times at ambient temperature. The vials should be opened and handled under a laminar flow cabinet to reduce the risk of contamination. The material is intended to be used to determine the cut-off values by the Standard Operating Procedures (SOPs) edited by the EURL-AP [9-15] for technical implementation of Regulation (EC) No 152/2009 [6].

The minimum sample intake is 4 µL.

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9 Annex A: Description of the dPCR method

The copy number concentrations have been determined by dPCR using the protocol described here. The primers and probes are those published by the EURL-AP for the qPCR protocol [15]. Volumes of 3.94 µL of IRMM-AD482a, IRMM-AD482b, IRMM-AD482c, respectively, were used undiluted in the dPCR assay. To analyse 12 replicate panels of the 12.765 digital Array ^{TM} IFC's (Fluidigm), a volume of 47.25 µL of the DNA sample was mixed with 72.75 µL of Pre-sample mix solution and 9 µL of this mixture was loaded per panel. The Pre-sample mix solution contained the primers and probes (2.25 µL each) for the ruminant target at final concentrations mentioned in the Table A1 together with 20x GE sample loading reagent (6 µL) and Universal Mastermix (Diagenode, Liège, Belgium) (60 µL) as recommended by the EURL-AP. The PCR was performed according to the specifications mentioned in Table A2. Then, the PCR runs then analysed with the Fluidigm Digital PCR Analysis V software using the following settings: quality threshold of 0.4; linear baseline correction; automatic Cq threshold method; target Cq range between 20 and 50.

Table A1: Primers and probes used to amplify the ruminant target in a simplex PCR reaction

PCR target	Primer/ probe	Sequence	Concentration in PCR reaction [nM]	Amplicon size [bp]
ruminant target	Forward primer	5'-CCA GCA TCA GAG TCT TTT CCA AAT-3'	440	85-86
	Reverse primer	5'-GAA GGA ATG ATG CTA AAG CTG AAA C-3'	440	
	Probe	FAM- 5'-CAA CTC TTC GCA TGA GGT GGC CAA A- 3' TAMRA	146	

Table A2: Thermal cycling protocol used for the simplex PCR reaction amplifying the ruminant target

Name	Phase	Time [s]	Temperature [°C]	Repeats
UNG and Hot start	UNG	120	50	1
	Hot start	600	95	
PCR cycles	Denaturation	15	95	50
	Annealing	60	60	

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Abstract
This report describes the processing of a set of plasmid solutions, IRMM-AD482a, b and c. The material was produced following ISO Guide 34:2009 [ ].
A DNA fragment specific for the identification of ruminant meat were cloned into a pUC18 vector to construct the pIRMM-0103 plasmid. The plasmid was diluted to three different concentration levels.
Between unit-homogeneity was quantified and stability during dispatch and storage were assessed in accordance with ISO Guide 35:2006 [ ].
The materials are intended for the determination of a cut-off value to discriminate positive samples from negative samples by quantitative PCR. As any reference material, the materials can also be used for control charts or validation studies. The RM is available as a set of three plastic tubes containing 1 mL of plasmid solution. The minimum amount of sample to be used is 4 µL.

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