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CERTIFICATION REPORT

A set of three plasmid DNA calibration solutions bearing a ruminant-specific DNA fragment

Certified Reference Materials: ERM®-AD482a, ERM®-AD482b, ERM®-AD482c

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European Commission
Joint Research Centre
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Abstract
This report describes the preparation and characterisation of a set of three plasmid solutions, ERM-AD482a, ERM-AD482b and ERM-AD482c. The material was produced in accordance with ISO Guide 34:2009.
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 of the entire pIRMM-0103 plasmid was determined by dye terminator cycle sequencing applying the primer walking method on the entire plasmid. The plasmid was put into a solution and its concentration was measured by ultraviolet (UV) spectrophotometry. Afterwards this solution was gravimetrically diluted to obtain three different plasmid concentration levels. The plasmid copy number concentration of the three concentration levels were certified by digital quantitative polymerase chain reaction methods (dPCR). In addition, between-unit homogeneity, as well as short-term long-term and freeze-thaw stability was assessed in accordance with ISO Guide 35:2006.
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 defined in the Standard Operating Procedure of the EU Reference Laboratory for animal proteins (EURL-AP) according to Commission Regulation No 51/2013.
As any certified reference material (CRM), the materials can also be used for control charts or validation studies. The CRM is available as a set of three vials each containing at least 1 mL of plasmid solution. The minimum amount of sample to be used is 4 µL.

CERTIFICATION REPORT

A set of three plasmid DNA calibration solutions bearing a ruminant-specific DNA fragment

Certified Reference Materials: ERM®-AD482a, ERM®-AD482b, ERM®-AD482c

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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 and characterisation of a set of three plasmid solutions, ERM-AD482a, ERM-AD482b and ERM-AD482c. The material was produced in accordance with 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 of the entire pIRMM-0103 plasmid was determined by dye terminator cycle sequencing applying the primer walking method on the entire plasmid. The plasmid was put into a solution and its concentration was measured by ultraviolet (UV) spectrophotometry. Afterwards this solution was gravimetrically diluted to obtain three different plasmid concentration levels. The plasmid copy number concentration of the three concentration levels were certified by digital quantitative polymerase chain reaction methods (dPCR). In addition, between-unit homogeneity, as well as short-term long-term and freeze-thaw stability was 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 defined in the Standard Operating Procedure of the EU Reference Laboratory for animal proteins (EURL-AP) according to Commission Regulation No 51/2013 [3, 4].

As any certified reference material (CRM), the materials can also be used for control charts or validation studies. The CRM is available as a set of three vials each containing at least 1 mL of plasmid solution. The minimum amount of sample to be used is 4 µL.

The following certified values were assigned:

	Copy number concentration of the plasmid	
	Certified value [cp/µL]	Uncertainty [cp/µL]
ERM-AD482a[1)]	123[2)]	30[4)]
ERM-AD482b[1)]	32[3)]	7[5)]
ERM-AD482c[1)]	8[3)]	3[5)]

[1)] Copy number concentration of the double stranded plasmid as measured by a digital PCR method amplifying a DNA fragment specific for ruminant tissue.

[2)] Unweighted mean value of 5 independent determinations. The value and its uncertainty are traceable to the International System of units (SI).

[3)] Unweighted mean value of 7 independent determinations. The value and its uncertainty are traceable to the International System of units (SI).

[4)] The uncertainty is an expanded uncertainty with a coverage factor k = 2.16 derived from an effective degrees of freedom of 13 and corresponding to a level of confidence of approximately 95 % estimated in accordance with ISO/IEC Guide 98-3, Guide to the Expression of Uncertainty in Measurement (GUM:1995), ISO, 2008 [5].

[5)] The uncertainty is an expanded uncertainty with a coverage factor k = 2 corresponding to a level of confidence of approximately 95 % estimated in accordance with ISO/IEC Guide 98-3, Guide to the Expression of Uncertainty in Measurement (GUM:1995), ISO, 2008 [5].

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Glossary

ANOVA	Analysis of variance
b	Slope in the equation of linear regression $y = a + bx$
bp	*Base pair*
BSE	Bovine spongiform encephalopathy
cp	Number of copies
Cq	Quantification cycle
CI	Confidence level
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
k	Coverage factor
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
PT	Proficiency test
qPCR	Quantitative polymerase chain reaction
dPCR	Digital 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) [6-8]. Regulation (EC) No 152/2009 of 27 January 2009 has laid down the methods of sampling and analysis for the official control of feed [9]. 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) [3]. The quantitative PCR (qPCR) methods are targeting remaining intact DNA fragments from ruminant origin that still can be detected in heavily processed feed samples.
Such a qPCR method has been recently validated by the EURL-AP in feeding stuffs. Calibrants are needed for the correct implementation of this qPCR 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 cycle (Cq) values obtained by qPCR are influenced by a large number of factors including the composition of the mastermix and the instrument used [10]. Therefore each control laboratory needs to determine its individual threshold value and verify this value on a regular basis according to its quality system. To determine a cut-off value, three plasmid solutions (each containing a defined copy number 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 such 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 [3].

1.2 Choice of the material

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

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 (more than 300 copies per cell) nuclear DNA fragment of 86 base pairs that can be amplified using two specific primers. The three calibrant solutions are certified for their absolute plasmid copy number concentration in the context of an inter-laboratory characterisation study.

1.3 Design of the project

EURL-AP provided the plasmid. At IRMM, the plasmid DNA was purified and linearized. The plasmid DNA concentration of the stock solution was measured by UV spectrophotometry and three solutions at nominal values of 128 cp/µL, 32 cp/µL and 8 cp/µL were prepared by gravimetric dilution of this stock solution. The concentrations of these three solutions have been verified by dPCR. The material has then been processed and a sequencing study, homogeneity, characterisation, freeze-thaw, short- and long-term stability studies 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)

2.3 Characterisation study

2.3.1 Sequence identity of the plasmid

Baseclear BV, Leiden, NL

Eurofins Madigenomix GmbH, Ebersberg, DE

2.3.2 Copy number concentration

European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (IRMM), Geel, BE

LGC Limited, Molecular and Cell Biology Team, Teddington, UK

National Measurement Institute (NMI), Department of Innovation, Industry, Science and Research, Bioanalysis Group, West Lindfield, AU

TATAA Biocenter AB, Göteborg, SE

National Institute of Biology, Ljubljana, SI

European Commission, Joint Research Centre, Institute for Health and Consumer Protection (IHCP), Ispra, IT

Centre Wallon de Recherches Agronomiques (CRA-W), Gembloux, BE

3 Material processing and process control

3.1 Origin and purity of the starting material

The pIRMM-0103 plasmid was received from the EURL-AP as a bacterial culture in Luria broth (LB) medium supplemented with glycerol. After plating it on LB Agar supplemented with ampicillin, a single colony was picked and cultured overnight in 5 mL LB medium supplemented with ampicillin. The plasmid was extracted from the bacterial culture using silica columns (Qiaquick Gel extraction kit, Qiagen, Venlo, The Netherlands) and resuspended in TE low buffer (1 mM Tris, 0.01 mM EDTA, pH 8).

3.1.1 Plasmid size confirmation

The size of the pIRMM-0103 plasmid was confirmed by restriction enzyme digestion followed by agarose gel electrophoresis.

3.1.2 Purity of the plasmid extract

In theory three different types of contaminants could be present in the plasmid extract: a) 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 plasmid; pIRMM-103 and one of the 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 *E. coli* 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 whilst the other plasmids will be 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 [11]. To ensure that the plasmid production started from a single bacterial clone, and would therefore contain only one type of plasmid, one additional plating step was included. A single colony containing the pIRMM-0103 was plated on a LB plate supplemented with ampicillin (50 µg/mL) 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, and it was therefore concluded that the plasmid preparation was not contaminated with large amounts of genomic DNA or RNA molecules from host bacterial cells, although, trace levels of either molecules cannot be detected by gel electrophoresis.

The linearised plasmid was analysed by spectrophotometry to detect the presence of non-nucleic acid contaminants e.g.: 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 [12]. 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 coding 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 trace levels of nucleic acids 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 which could be present in the final plasmid preparation. Traces of chemical residues from nucleic acid extraction like phenol and guanidine might, however, partially 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 [13].

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. Since the plasmid concentration measured by UV spectrophotometry is very similar to the plasmid concentration estimated on by dPCR, it can be concluded that the undiluted stock solution does not contain major impurities.

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 to generate the final plasmid solutions, it can be concluded that there are no major contaminations present in the plasmid solution.

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 (*Zea mays*) genomic DNA was isolated and purified from untreated (non-GM) 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 autoclaving cycles to fragmentise the DNA. The fragmentation was confirmed by gel electrophoresis.

The plasmid solution was diluted gravimetrically to 1280 cp/µL in 1 mmol/L Tris, 0.01 mmol/L EDTA pH 8.0 supplemented with 12 ng maize DNA/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 and cooled conditions. Once filled, the vials were closed manually, placed in boxes that were then closed and stored at -20 °C. The kits were then assembled into a plastic box containing one vial of each concentration level and stored back at -20 °C. At no point after the initial freezing were the vials allowed to thaw.

3.3 Process control

Before each dispensing, each indicative final plasmid concentration was verified by dPCR as described in Annex A.

4 Homogeneity of the copy number concentration of the plasmid

A key requirement for any reference material aliquotted into units is the equivalence between those units, ensuring that the certified value of the CRM is valid for all vials, within the stated uncertainty. ISO Guide 34 requires RM producers to quantify the between-unit variation. This aspect is covered by performing between-unit homogeneity studies.

4.1 Between-unit homogeneity

The study was carried out to control the gravimetric dilutions of the three plasmid solutions. The measurements were performed under repeatability conditions on vials randomly taken from the entire batch and analysed in a randomised order. The between-unit homogeneity was evaluated for each of the 3 concentration levels of ERM-AD482.

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 12.765 Array ™ IFC's (Fluidigm). The measurements were performed under repeatability conditions in a randomised manner which allowed separating a potential analytical trend from a possible trend in the filling sequence.

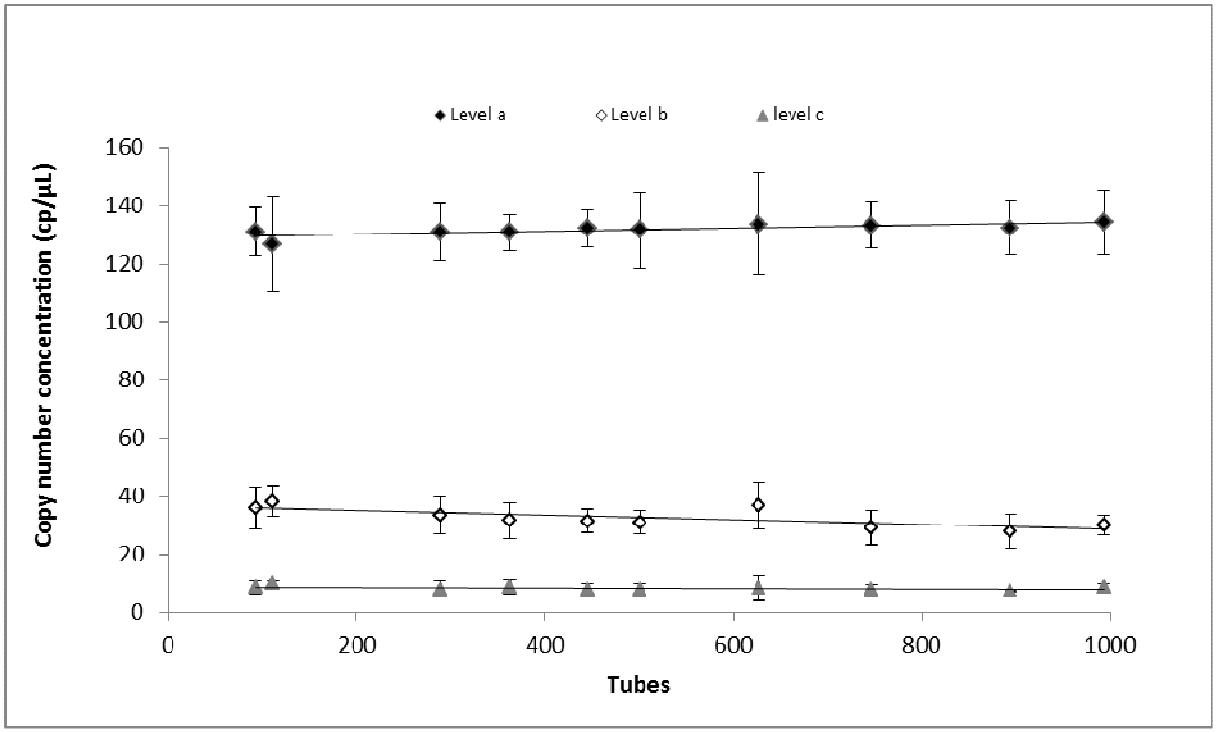

Figure 1: Copy number concentrations measured by dPCR for level a, b and c batches. Each point represents the average of 5 measurements; bars indicate the standard deviation at 95 % CI.

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 95 %. A filling trend was detected for ERM-AD482a, however, no filling trend was observed for and ERM-AD482b and ERM-AD482c (Figure 1). The filling trend observed for ERM-AD482a will not affect the use of the CRM kit.

The dataset was tested for consistency using Single Grubbs outlier tests on a CI of 99 % on the individual results and the unit means. No outlying individual results and no outlying unit means were detected.

For ERM-AD482b and ERM-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.

It has to 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 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.* [14]. 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 ERM-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 ERM-AD482a, a more conservative approach was used to estimate the uncertainty related to homogeneity. As the trend in the filling sequence was significant at 95 % 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:

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

The results of the evaluation of the between-unit variation are summarised in Table 2. The resulting values from the above equations were converted into relative uncertainties.

For ERM-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 ERM-AD482b and ERM-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 2: Results of the between-unit homogeneity study

Material	$s_{wb,rel}$ [%]	$s_{bb,rel}$ [%]	$u^*_{bb,rel}$ [%]	$u_{rec,rel}$ [%]	$u_{bb,rel}$ [%]
ERM-AD482a	n.a.	n.a.	n.a.	10.15	10.15
ERM-AD482b	17.99	6.92	3.81	n.a.	6.92
ERM-AD482c	27.42	n.c.[1)]	5.80	n.a.	5.80

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

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 the 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 [15] and under repeatability conditions which greatly improves the sensitivity of the stability tests. In that approach, samples were stored for a specified length of 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 each of 0, 1, 2 and 4 weeks whereupon they were moved to the reference temperature (-70 °C). Samples were stored at two testing temperature for safety reasons. Storage temperature at -20 °C is the expected shipping temperature. Storage temperature +4 °C is tested to check the stability of the material also above the transportation temperature, to cover transportation scenarios where the material might get exposed to elevated temperatures. 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 trend 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 3.

Table 3: Results of the short-term stability study, dPCR, u_{sts} provided at 1 week

Material	Number of individual outlying results at 99 % CI		Significance of the trend at 95% CI	
	+4 ºC	-20 ºC	+4 ºC	-20 ºC
ERM-AD482a	none	none	yes	no
ERM-AD482b	none	1 (statistically retained)	no	no
ERM-AD482c	none	none	no	no

One statistical outlier was detected for ERM-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 95 % CI at -20 °C. Based on these data the material shall be shipped frozen on dry ice, however even at 4 °C it is stable enough.

5.2 Freeze-thaw study

A typical sample intake in a qPCR test is five µL. 15 µL of each calibration solution are needed to establish a calibration curve with 3 replicates per concentration level. As each vial contains at least 1000 µL, a minimum of 65 calibration curves can be prepared with one kit. As the storage temperature is -20 °C, repeated use of the CRM will require repeated freeze-thaw cycles. The effect of repeated freeze-thaw cycles was therefore investigated in this freeze-thaw study.

An approach similar to the isochronous design was followed out. Vials from ERM-AD482a, ERM-AD482b and ERM-AD482c were analysed. For each concentration, two vials were exposed to 0, 8, 16 and 32 freeze-thaw cycles and moved to the reference temperature (-20 °C) afterwards.

Each vial was measured once, with simplex real time dPCR as described in Annex A. The measurements were done under intermediate precision conditions.

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. The slope of the regression line were not significantly different from zero (95 % CI) for ERM-AD482b. For ERM-AD482a and ERM-AD482c the slope of the regression line was significantly different from zero (95 % CI), after 8 freeze-thaw cycles (Figure 2). Additionally, a closer investigation of the results for the test vials showed that there was a significant degradation after 8 cycles in ERM-AD482a and ERM-AD482c.

Due to the observed degradation the CRMs should not be exposed to more than 5 freeze-thaw cycles.

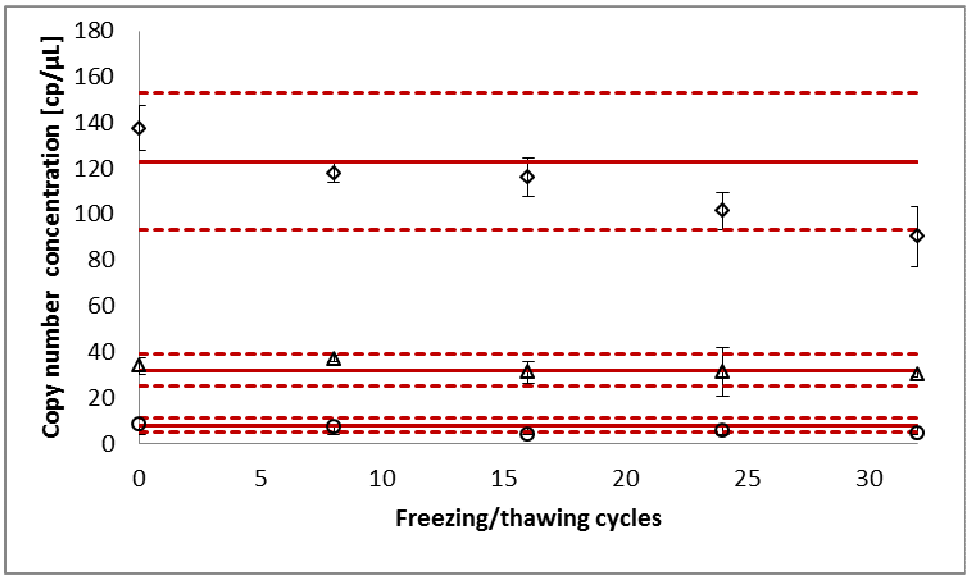

Figure 2: Copy number concentration of the ruminant target for ERM-AD482a (♦), ERM-AD482b (Δ) and ERM-AD482c (o) upon successive freeze-thaw cycles. The bars represent the standard deviation of 2 investigated vials per test freeze-thaw cycle at 95 % CI.The red solid lines indicate the respective certified values and the dashed lines represent the respective certified values +/- the expanded uncertainties.

5.3 Long-term stability study

Long-term stability (LTS) study, i.e. storage conditions evaluation of ERM-AD482a, b, c was performed by storing the samples at -20 °C for 0, 2, 4 and 6 months. Five units per storage time were selected using a random stratified sampling scheme. Each unit was measured once, on one panel of a 12.765 digital array using dPCR. The measurements were performed under repeatability conditions and in a randomised sequence to be able to separate a potential analytical trend 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. Two outlying individual results were found in the ERM-AD482c at -20 °C. As no technical reason for the outlier could be found all data were retained for statistical analysis and for the estimation of uncertainty of LTS (u_{LTS}). None of the trends was statistically significant on a 95 % CI at -20 °C. The results of the statistical evaluation of the long-term stability are summarised in Table 4.

Table 4: Results of the long-term stability study

Material	Number of individual outlying results at 99% CI	Significance of the trend at 95% CI
	-20 ºC	-20 ºC
ERM-AD482a	none	no
ERM-AD482b	none	no
ERM-AD482c	2 (statistically retained)	no

The data were plotted against storage time and regression lines of copy number concentration versus time were calculated, as shown in Figure 4. The slope of the regression lines was then tested for statistical significance (95 % confidence level). A positive trend was observed for ERM-AD482c. As the analyte cannot be created in the sample, a positive trend could only be due to degradation of the matrix. This, however, should be seen for all concentration levels, which is not

the case. The observed trend was therefore regarded as an artefact. As it is also shown by Figure 3, the regression line runs within the calculated, combined, expanded uncertainties. The uncertainty contribution of LTS was calculated at 18 months and the values are shown in Table 5.

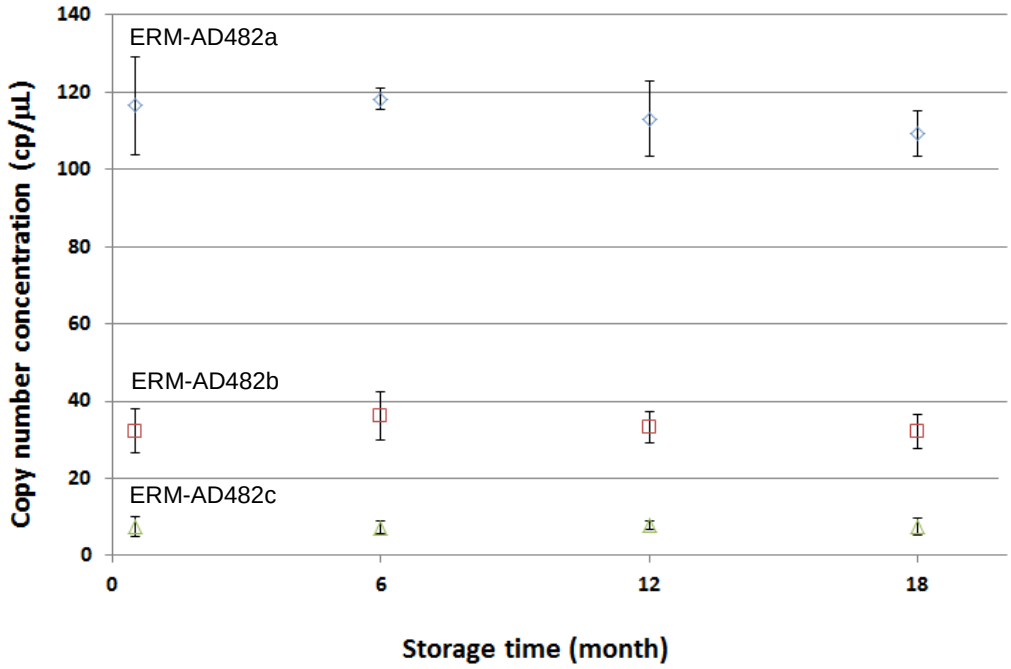

Figure 3: Stability of ERM-AD482c (green), b (red) and a (blue) over 18 months. The averages were calculated over n=5.

Table 5: Uncertainty contribution of the long term stability for a shelf life of 18 months

Material	u_{LTS} (%)
ERM-AD482a	4.23
ERM-AD482b	7.61
ERM-AD482c	13.26

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

6 Characterisation study

Material characterisation is the process of determining the property value, i.e. the plasmid copy number concentration of the reference material.

6.1 Characterisation study for the sequence identity

The structural identity of the plasmid i.e. the sequence of pIRMM-0103 was determined by dideoxy terminator sequencing (or Sanger sequencing) using the primer walking method.

6.1.1 Study set up

The selection of two nucleic acids sequencing companies was based on criteria that comprised aspects of both technical competence and quality management aspects. Both companies were required to operate a quality system and to deliver documented evidence of its laboratory proficiency in the field dideoxy terminator sequencing. Holding a formal accreditation was not mandatory, but meeting the requirements of ISO/IEC 17025 was obligatory.

Both companies have received each one vial containing 15 µL of circular pIRMM-0103 plasmid dissolved in TE buffer, which vials had been set aside during ERM-AD482 processing. The companies were commissioned to perform double stranded sequencing of an entire circular plasmid by using primer walking. Sequences were generated using the BigDye® Terminator chemistry version 3.0 (ABI). The sequence reactions were analysed on ABI3730 capillary sequencers.

6.1.2 Results

Each laboratory provided a full sequence of the pIRMM-0103 plasmid. Comparison of both sequences showed that they were identical and except for 1 nucleotide, identical to the sequence provided by EURL-AP. This one nucleotide was reported as uncertain, in parenthesis, by EURL-AP. It is a thymine, at position 1238, in the pCR2.1 cloning site, as shown by Annex B. It is not part of the amplified sequence, which is indicated in yellow for the primer and in cyan for the probe binding sites and the one base mismatch is therefore irrelevant. One laboratory also delivered the sequence quality values allowing to calculate the highest cumulative error rate for the entire plasmid which remains marginal ($1/6.57801 \times 10^{8}$).

6.2 Characterisation study for the copy number concentration of the plasmid

The material characterisation was based on an interlaboratory comparison study carried out by expert laboratories (see section 2.3.2). The certified value for the copy number concentration of the three plasmid dilutions was determined by dPCR measurements performed by seven different laboratories. All the seven participants used the BioMark system (Fluidigm) and the 12.765 digital arrays to perform the measurements. This study design results in the randomisation of laboratory bias, thereby reducing the combined uncertainty.

6.2.1 Study set up

The seven laboratories were selected based on criteria that comprised aspects of 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 the field of dPCR measurements by previous publications. The need of a formal accreditation was not mandatory, but meeting the requirements of ISO/IEC 17025 was obligatory.

Each laboratory received two vials of each plasmid concentration of ERM-AD482 and was commissioned to provide two sets of results, one set per vial. Each vial was analysed on five panels of one digital array. For the whole study 6 digital arrays were used per laboratory. The ruminant target was amplified using the conditions shown in Annex A. Vials for material characterisation were selected using a random stratified sampling scheme covering the whole batch. Each of the two vials of each concentration was measured on different days and on different digital arrays to ensure intermediate precision conditions were met.

6.2.2 PCR conditions for the characterisation study

As the characterisation studies of ERM-AD482 and ERM-AD483 (a calibration kit for the detection of PAPs of porcine origin) were performed in parallel on the same plate, an annealing temperature of 50°C was chosen for both assays. Previous verification of the assay showed no significant variation in the copy number concentration estimation when varying the annealing temperatures, from 50 ºC to 60 ºC, indicating that the dPCR assay is robust.

6.2.3 Certified value

The characterisation campaign resulted in, 7 independent datasets. 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:

- compliance with the analysis protocol
- correctness of the gravimetric measurements and records
- visual check if there was no air bubble present in the panels
- each independent measurement result based on the results of 5 panels
- measurements of the 2 vials per candidate reference material were done on different days
- profile of the amplification curves, i.e.: smeared vs. sharp

Based on the above, for ERM-AD482a two entire datasets were rejected as technically invalid, due to the partially inhibited amplification profiles (data not shown). To quantify in which extent the amplification was inhibited, we counted the percentage of amplification curves that reached a relative fluorescence level (ΔRN) of at least 0.65. If less than 95 % of the amplification curves reached that level, the panel was flagged. If two or more panels out of the five were flagged, the chip was excluded from analysis as the assay was considered as suboptimal. Hence, the certified value for ERM-AD482a was calculated on 5 datasets. The degree of freedom associated with the estimation of uncertainty for ERM-AD482a has been calculated using the Welch–Satterthwaite equation [5]. This equation provides an approximation of the effective degrees of freedom of a linear combination of the independent sample variances. The variances were gathered from the homogeneity study, stability studies and the characterisation study (Annex D). For ERM-AD482, an effective degrees of freedom (ν_{eff}) of 13 was calculated, providing a coverage factor *k* of 2.16. The datasets accepted on technical grounds 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 99 % confidence level). The dataset means follow a normal distribution. None of the data contains outlying means. The datasets are therefore consistent and the mean of independent measurement results is a good estimate of the true value.

The uncertainty for the characterisation exercise was estimated as the relative standard uncertainty of the independent measurement results, i.e. $\frac{s_{all\ measurements}}{\sqrt{n}}$ with *s* the relative standard deviation of the technically valid independent measurements results and *n* the number of technically valid independent determinations. The results of these evaluations are shown in Table 6.

Table 6: Statistical evaluation of the technically accepted data sets for the characterisation of ERM-AD482

Copy number concentration of the plasmid									
CRM	*n*	*k*	Outliers		Normally distributed	Statistical parameters			
			Means	Variances		Mean [cp/µL]	*s* [cp/µL]	u_{char} [cp/µL]	$u_{char, rel}$ [%]
ERM-AD482a	5	2.16	none	none	yes	122.61	4.94	2.21	1.80
ERM-AD482b	7	2	none	none	yes	31.67	3.40	1.29	4.06
ERM-AD482c	7	2	none	none	yes	8.00	1.15	0.43	5.41

Legends: *n* number of technically valid independent determinations, *k* is the coverage factor, *s* standard deviation, u_{char} uncertainty related to the characterisation study and $u_{char,\ rel}$ is the relative uncertainty related to the characterisation study

7 Value Assignment

Certified values were assigned. Certified values are values that fulfil the highest standards of accuracy. Procedures at IRMM generally require pooling of not less than 6 datasets to assign certified values. Full uncertainty budgets in accordance with the 'Guide to the Expression of Uncertainty in Measurement' [5] were established. ERM-AD482 was certified for its copy number concentration by dPCR.

7.1 Certified value and its uncertainty

The unweighted mean of the means of the accepted datasets as shown in Table 7 was assigned as certified value.

The assigned uncertainty consists of uncertainties relating to potential between-unit inhomogeneity, u_{bb} (Section 4.1), to potential degradation during short storage u_{sts} (Section 5.1), to potential degradation during long-term storage, u_{lts} (Section 5.3) and to characterisation, u_{char} (Section 6.2).

These different contributions were combined to estimate the relative expanded uncertainty of the certified value ($U_{CRM,\ rel}$) with a coverage factor *k* as:

$$U_{CRM,rel} = k \cdot \sqrt{u_{bb,rel}^2 + u_{sts,rel}^2 + u_{lts,rel}^2 + u_{char,rel}^2} \qquad \textbf{Equation 6}$$

- $u_{bb,rel}$ was estimated as described in Section 4.1
- $u_{sts,rel}$ was estimated as described in Section 5.1
- $u_{lts,rel}$ was estimated as described in Section 5.3
- $u_{char,rel}$ was estimated as described in Section 6.2

Because of the sufficient numbers of the degrees of freedom of the different uncertainty contributions for dPCR, a coverage factor *k* of 2 was applied for ERM-AD482b and c, to obtain the expanded uncertainty. In case of ERM-AD482a, since only 5 independent datasets could be retained, a coverage factor *k* = 2.16 was applied.

The certified value and its uncertainty are summarised in Table 7.

Table 7: Certified value and its uncertainty for ERM-AD482 using dPCR.

	Certified value [cp/µL]	$u_{bb,\ rel}$ [%]	$u_{sts,\ rel}$ [%]	$u_{lts,\ rel}$ [%]	$u_{char,\ rel}$ [%]	$u_{CRM,\ rel}$ [%]	U_{CRM} [1)] [cp/µL]
ERM-AD482a	123	10.15	1.19	4.23	1.80	24.20	30
ERM-AD482b	32	6.92	2.92	7.61	4.06	22.87	7
ERM-AD482c	8	5.80	5.57	13.26	5.41	32.85	3

[1)] expanded and rounded uncertainty, using *k* = 2,16 for ERM-AD482a and *k* = 2 for ERM-AD482b and c

8 Metrological traceability and applicability of ERM-AD482

8.1 Metrological traceability

8.1.1 Quantity value and identity of the measurand

The DNA copy number concentration and identity of ERM-AD482 is a method-defined measurand and has been obtained by dPCR using primers, probe and PCR conditions shown in Annex A, Table A1. Traceability of the 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 SI. Consistency within each of the inter-laboratory comparisons demonstrates that all relevant input factors were covered. As the assigned values are combinations of agreeing results, individually traceable to the SI, the assigned quantity values themselves are also traceable to the SI.

8.1.2 Sequence of the plasmid

The sequence of the plasmid was confirmed by two expert sequencing service companies using dye terminator cycle sequencing applying the primer walking method on the entire plasmid. The participants used different methods for the sample preparation as well as for the final determination of the sequence, demonstrating absence of measurement bias. The sequence of the plasmid is therefore structurally defined and independent of the measurement method.

In conclusion, the measurand is method-defined as the certified value was measured by dPCR.

9 Instructions for use

9.1 Safety information

The usual laboratory safety measures apply.

9.2 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 sunlight.

Assuming that major contamination during handling of opened vials is excluded, the solutions can be used for several experiments. The material should however not undergo more than 5 freeze-thaw cycles and therefore it is recommended that the solutions are aliquotted immediately at first use. Additional vortexing of the material is not recommended, and should be avoided The material can also be stored at +4°C for a total maximum period of 1 month 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 which may occur during storage of the material at the customer's premises, especially of opened vials.

9.3 Preparation and use of the material

To prepare 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 [4, 18] for technical implementation of Regulation (EC) No 152/2009 [9].

The minimum sample intake is 4 µL.

10 Acknowledgments

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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 [18]. Volumes of 4 µL of ERM-AD482a, ERM-AD482b, ERM-AD482c, respectively, were used undiluted in the dPCR assay. To analyse 12 replicate panels of the 12.765 digital Array ™ IFC's (Fluidigm), the DNA sample was mixed with the Pre-sample mix solution and 8 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	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[1]	

[1)] for the characterisation study an annealing temperature of 50 °C was chosen for practical reason.

Annex B: Sequence alignment of pIRMM-0103

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Seq1       AGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTT 50
Seq2       AGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTT 50
Seq3       AGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTT 50

Seq1       TTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCG 100
Seq2       TTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCG 100
Seq3       TTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCG 100

Seq1       GTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAAC 150
Seq2       GTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAAC 150
Seq3       GTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAAC 150

Seq1       TGGCTTCAGCAGAGCGCAGATACCAAATACTGTTCTTCTAGTGTAGCCGT 200
Seq2       TGGCTTCAGCAGAGCGCAGATACCAAATACTGTTCTTCTAGTGTAGCCGT 200
Seq3       TGGCTTCAGCAGAGCGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGT 200

Seq1       AGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCT 250
Seq2       AGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCT 250
Seq3       AGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCT 250

Seq1       CTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCT 300
Seq2       CTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCT 300
Seq3       CTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCT 300

Seq1       TACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGG 350
Seq2       TACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGG 350
Seq3       TACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGG 350

Seq1       GCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTAC 400
Seq2       GCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTAC 400
Seq3       GCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTAC 400

Seq1       ACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCC 450
Seq2       ACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCC 450
Seq3       ACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCC 450

Seq1       CGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAG 500
Seq2       CGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAG 500
Seq3       CGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAG 500

Seq1       GAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGT 550
Seq2       GAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGT 550
Seq3       GAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGT 550

Seq1       CCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTC 600
Seq2       CCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTC 600
Seq3       CCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTC 600

Seq1       GTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTAC 650
Seq2       GTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTAC 650
Seq3       GTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTAC 650

Seq1       GGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTA 700
Seq2       GGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTA 700
Seq3       GGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTA 700

Seq1       TCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATAC 750
Seq2       TCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATAC 750
Seq3       TCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATAC 750

Seq1       CGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAG 800
Seq2       CGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAG 800
Seq3       CGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAG 800

Seq1       CGGAAGAGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATT 850
Seq2       CGGAAGAGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATT 850
Seq3       CGGAAGAGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATT 850
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Seq3        CATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGA 900

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Seq2        GCGCAACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTT 950
Seq3        GCGCAACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTT 950

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Seq2        TACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAA 1000
Seq3        TACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAA 1000

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Seq2        CAATTTCACACAGGAAACAGCTATGACCATGATTACGAATTCGAGCTCGG 1050
Seq3        CAATTTCACACAGGAAACAGCTATGACCATGATTACGAATTCGAGCTCGG 1050

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Seq3        AGCTGTGACCGTCTCCGGGAGCTGCATGTGTCAGAGGTTTTCACCGTCAT 1650

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Seq3       GAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGA 2050

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Seq2       ACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTAT 2099
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Seq1       TATCCCGTATTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTAT 2149
Seq2       TATCCCGTATTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTAT 2149
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Seq3       GGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTG 2250

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Seq3       ATAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAG 2300

Seq1       CTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTTGATCG 2349
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Seq3       CTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTTGATCG 2350

Seq1       TTGGGAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCA 2399
Seq2       TTGGGAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCA 2399
Seq3       TTGGGAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCA 2400

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Seq2       CGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAA 2449
Seq3       CGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAA 2450

Seq1       CTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGA 2499
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Seq3       CTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGA 2500

Seq1       TAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTA 2549
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Seq1       GCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGAC 2649
Seq2       GCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGAC 2649
Seq3       GCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGAC 2650

Seq1       GGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAG 2699
Seq2       GGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAG 2699
Seq3       GGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAG 2700

Seq1       GTGCCTCACTGATTAAGCATTGGTAACTGTCAGACCAAGTTTACTCATAT 2749
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Seq3       GTGCCTCACTGATTAAGCATTGGTAACTGTCAGACCAAGTTTACTCATAT 2750

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Seq2       GAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTT 2849
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Seq1       CGTTCCACTG 2859
Seq2       CGTTCCACTG 2859
Seq3       CGTTCCACTG 2860
```

Legend: Seq1 is provided by Eurofins, Seq2 is provided by BaseClear, and Seq3 is an *in silico* sequence provided by EURL-AP.

Annex C: Raw data of the characterisation study

Table A3: Raw data of the five characterisation data sets for of ERM-AD482a

Labs	Mean	STDev	H.W. CI (95%)	Samp#1	Samp#2	Samp#3	Samp#4	Samp#5	Samp#6	Samp#7	Samp#8	Samp#9	Samp#10
L0 - Coordinator	130.664	15.3382	10.9723	146.29	137.09	127.14	142.29	159.6	123.59	110.6	119.1	125.61	115.33
L1 -Lab 1	-	-	-	-	-	-	-	-	-	-	-	-	-
L2 -Lab 2	117.63	8.5346	6.1053	129.39	124.17	129.39	121.09	120.98	114.17	107.5	110.55	112.45	106.61
L3 -Lab 3	-	-	-	-	-	-	-	-	-	-	-	-	-
L4 -Lab 4	122.848	8.1642	5.8403	117.49	113.68	121.03	108.69	124.5	130.51	136.01	123.54	123.25	129.78
L5 -Lab 5	119.874	9.6885	6.9307	118.89	129.56	117.68	132.5	100.74	127.35	114.32	114.78	128.6	114.32
L6 -Lab 6	122.034	7.1805	8.9158	125.8	123.02	117.21	112.91	131.23	-	-	-	-	-

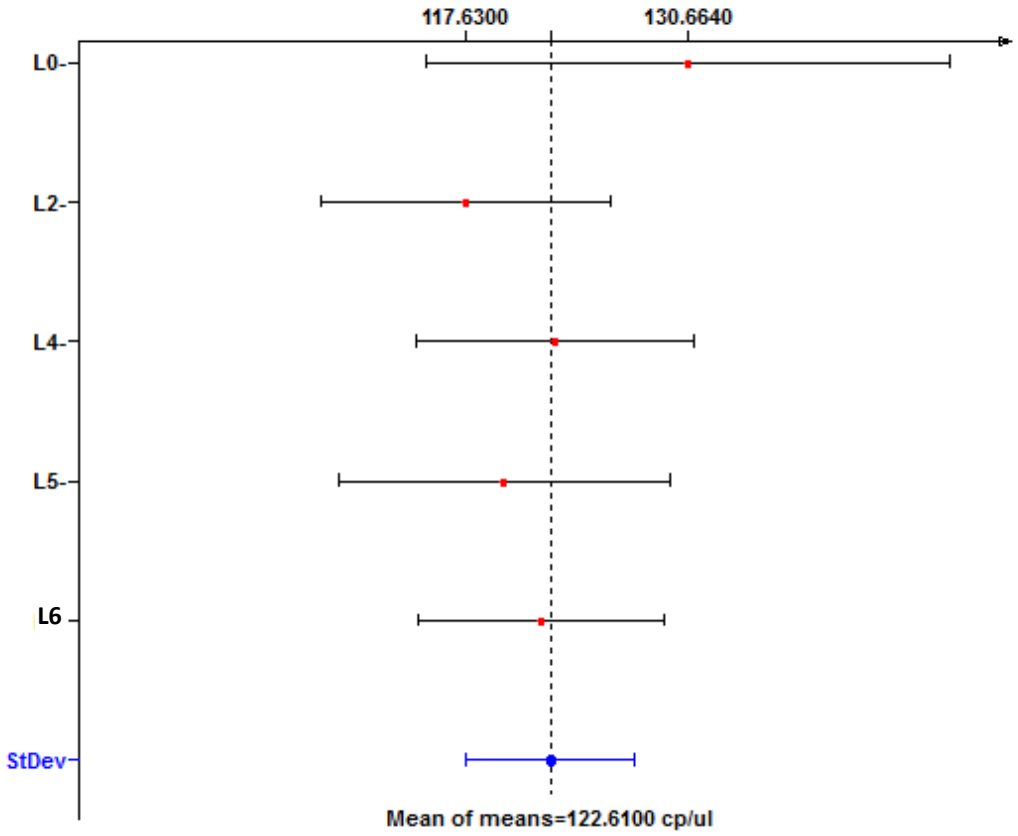


Table A4: Raw data of the seven characterisation data sets for of ERM-AD482b

Labs	Mean	STDev	H.W. CI (95%)	Samp#1	Samp#2	Samp#3	Samp#4	Samp#5	Samp#6	Samp#7	Samp#8	Samp#9	Samp#10
L0 - Coordinator	35.391	4.2663	3.0519	38.96	25.18	34.43	31.63	38.71	37.31	37.86	35.56	35.58	38.69
L1 -Lab 1	28.124	3.3256	2.379	26.38	29.19	29.26	32.39	25.97	23.14	31.22	28.88	31.6	23.21
L2 -Lab 2	30.851	4.6621	3.3351	27.69	35.33	33.47	28.46	26.38	25.41	33.74	25.31	35.01	37.71
L3 -Lab 3	27.89	4.6697	3.3405	35.44	19.48	29.34	27.96	30.76	30.11	27.51	21.19	26.64	30.47
L4 -Lab 4	36.5	8.653	6.19	40.67	27.73	33.49	36.68	30.4	38.64	58.08	29.83	37.22	32.26
L5 -Lab 5	29.953	3.2786	2.3454	24.79	34.61	27.49	32.37	25.85	29.2	32.77	30.07	33.2	29.18
L7 -Lab 6	32.994	5.9242	4.2379	27.42	35.93	37.16	33.05	27.42	45.5	34.09	27.34	34.91	27.12

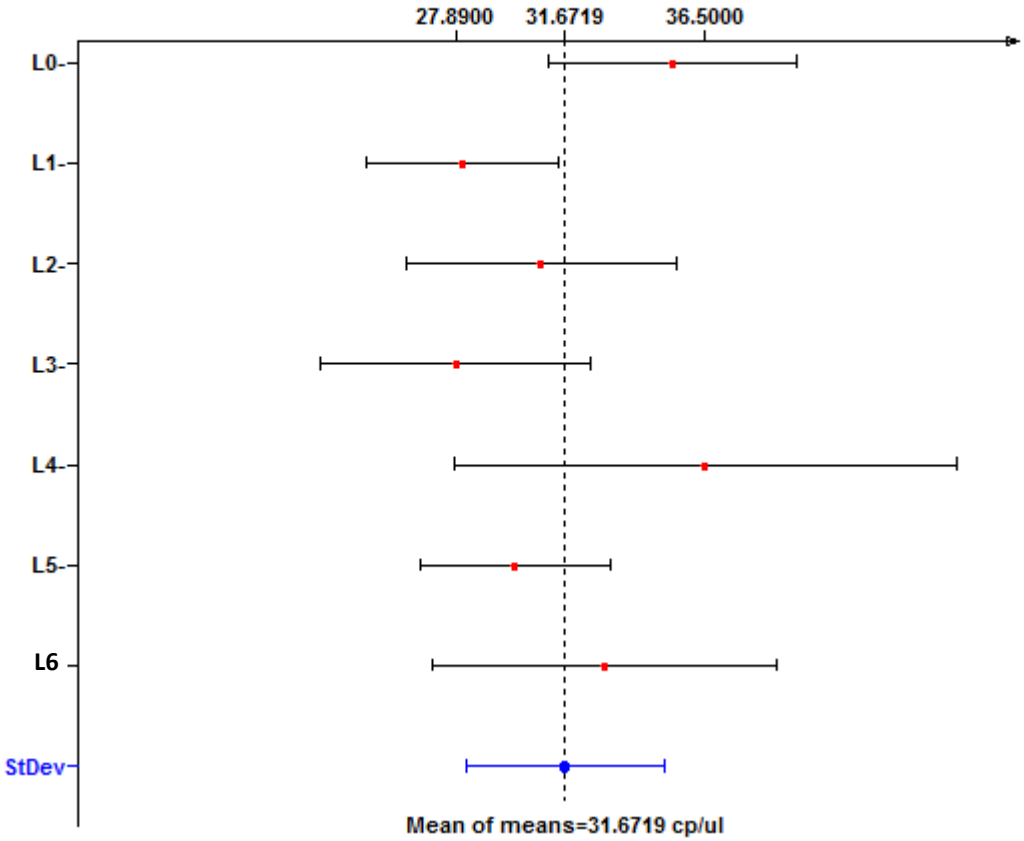
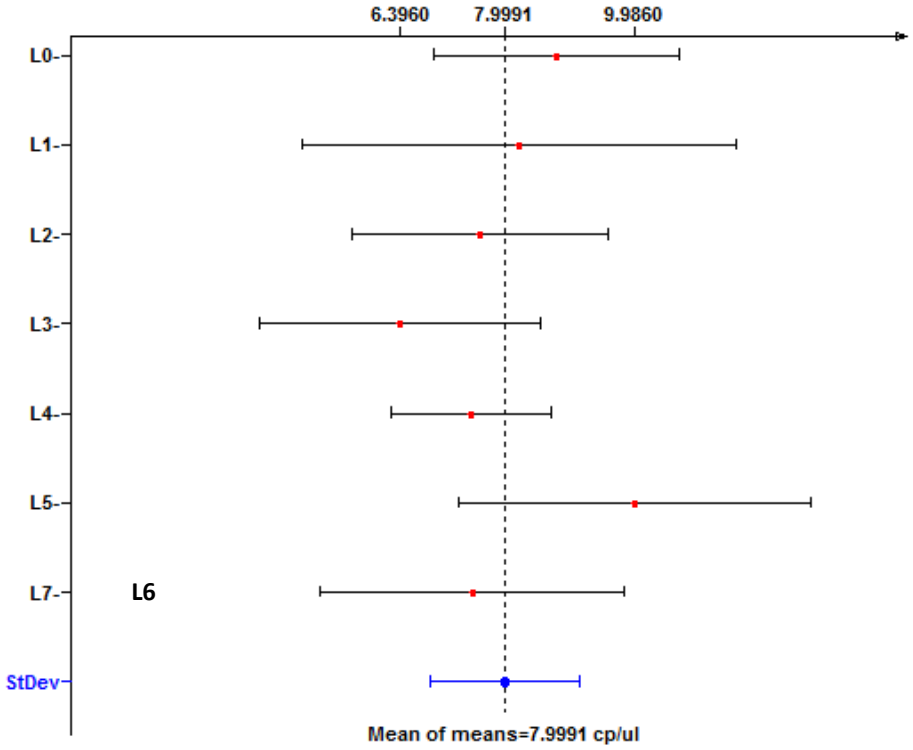


Table A5: Raw data of the seven characterisation data sets for of ERM-AD482c

Labs	Mean	STDev	H.W. CI (95%)	Samp#1	Samp#2	Samp#3	Samp#4	Samp#5	Samp#6	Samp#7	Samp#8	Samp#9	Samp#10
L0 - Coordinator	8.792	1.8882	1.3508	7.51	6.53	8.14	6.81	12.16	10.41	10.09	10.43	8.67	7.17
L1 -Lab 1	8.23	3.323	2.3771	8.29	5.47	12.71	4.42	3.87	13.62	7.65	10.31	9.45	6.51
L2 -Lab 2	7.611	1.9594	1.4017	8.8	6.85	8.89	3.77	5.63	7.68	7.88	7.1	10.92	8.59
L3 -Lab 3	6.396	2.1446	1.5341	8.34	3.74	5.01	4.5	6.09	9.23	4.97	4.56	9.17	8.35
L4 -Lab 4	7.48	1.2246	0.876	7.76	6.17	8.35	5.98	7.54	7.07	7.73	8.26	9.89	6.05
L5 -Lab 5	9.986	2.6879	1.9228	14.19	8.35	8.31	7.57	15.44	8.69	10.66	8.8	8.43	9.42
L7 -Lab 6	7.499	2.3337	1.6694	9.22	8.39	4.35	7.13	9.64	9.45	10.81	4.29	5.58	6.13



Annex D: Effective degrees of freedom and obtained *k* value for ERM-AD482a

When performing uncertainty analysis, it is important to calculate the degrees of freedom associated with the estimation of uncertainty. The Welch–Satterthwaite equation has been used to calculate an approximation to the effective degrees of freedom of a linear combination of the independent sample variances. The variances were gathered from the homogeneity study, stability studies and the characterisation study.

Table A4: Calculation of the effective degrees of freedom for ERM-AD482a

effective df from Welch-Satterthwaite for concentration level a							
Uncertainty components	**u**	**degrees of freedom (df)**					
Comb. relative	11.21						
Homogeneity (h)	10.15	9		between unit degrees of freedom			
STS	1.19	14		# determinations- 2			
LTS	4.23	43		# determinations- 2			
Characterisation (c)	1.8	4		5 labs -1			
Calculation:							
uc4 (y)	15768.2016122500			U comb relative			
ui4 (h) / df	1179.292834			u homogeneity			
ui4 (STS) / df	0.143238515			u short term stability			
ui4 (LTS) / df	7.445485358			u long term stability			
ui4 (c) / df	2.6244			u characterisation			
sum	1189.505958						
df effective:	13.25609301	rounded to 13					
from Table G.2 of GUM 2008, at p = 95% :			k = 2.16				

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Author(s): P. Corbisier, J. Mátrai, S. Mazoua, A.M. Kortekaas, P.Y.J. Chung, E. Scaravelli, S. Trapmann, H. Emons
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