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CERTIFICATION REPORT

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

Certified Reference Materials: ERM®-AD483a, ERM®-AD483b, ERM®-AD483c

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Abstract
This report describes the preparation and characterisation of a set of plasmid solutions, ERM-AD483a, ERM-AD483b and ERM-AD483c. The materials were produced in accordance with ISO Guide 34:2009.
A DNA fragment specific for the identification of a porcine target was cloned into a pUC18 vector to construct the pIRMM-0104 plasmid. The nucleic acid sequence of the entire pIRMM-0104 plasmid was determined by dye terminator cycle sequencing. The plasmid was eluted in TElow buffer, and its concentration was measured by ultraviolet (UV) spectrophotometry. Afterwards, it was gravimetrically diluted to three different concentration levels. The plasmid copy number concentration of the three concentration levels were certified by digital and droplet digital quantitative polymerase chain reaction methods, dPCR and ddPCR respectively. In addition, between-unit homogeneity, as well as short-term and long-term 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 porcine 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 (EU) No 51/2013.

As any certified reference material (CRM), ERM-AD483 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.

ERM
European Reference Materials

CERTIFICATION REPORT

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

Certified Reference Materials: ERM®-AD483a, ERM®-AD483b, ERM®-AD483c

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Summary

This report describes the preparation and characterisation of a set of plasmid solutions, ERM-AD483a, ERM-AD483b and ERM-AD483c. The materials were produced in accordance with ISO Guide 34:2009 [1].

A DNA fragment specific for the identification of a porcine target was cloned into a pUC18 vector to construct the pIRMM-0104 plasmid. The nucleic acid sequence of the entire pIRMM-0104 plasmid was determined by dye terminator cycle sequencing. The plasmid was eluted in TE_{low} buffer, and its concentration was measured by ultraviolet (UV) spectrophotometry. Afterwards, it was gravimetrically diluted to three different concentration levels. The plasmid copy number concentration of the three concentration levels were certified by digital and droplet digital quantitative polymerase chain reaction methods, dPCR and ddPCR respectively. In addition, between-unit homogeneity, as well as short-term and long-term 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 porcine 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 (EU) No 51/2013 [3, 4].

As any certified reference material (CRM), ERM-AD483 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-AD483a[1)]	126[2)]	18[3)]
ERM-AD483b[1)]	34[2)]	6[3)]
ERM-AD483c[1)]	9[2)]	3[3)]

[1)] Copy number concentration of the double stranded plasmid as measured by a digital PCR (dPCR) and droplet digital PCR (ddPCR) methods amplifying a DNA fragment specific for porcine.
[2)] The unweighted mean value of 7 independent determinations obtained by dPCR and by ddPCR. The value is traceable to the International System of units (SI).
[3)] 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
cp	Number of copies
BSE	Bovine spongiform encephalopathy
Cq	Quantification threshold
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
k	Coverage factor
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_{eff}	Number of effective replicates per unit, when per unit, the number of replicates is not the same.
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
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 when appropriate
s_{meas}	Standard deviation of measurement data; an additional index "rel" is added when 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_{low}	Buffer containing TRIS, 0.01 mmol/L EDTA at pH 8.0
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) as feed ingredients for farmed animals and in aquacultures, is regulated within the European Union through several regulations (Regulation (EC) 999/2001, Regulation (EC) 1774/2002 and Regulation (EC) 1234/2003) [4, 6-8]. In 2001, all PAPs were banned from the feed, and only in 2013 was this ban lifted for non-ruminant PAPs to be used in aquacultures [9]. Moreover, Regulation (EC) 1774/2002 also bans intra-species recycling [7]. Following these regulations, methods for species-specific detection of PAPs in compound feeds is required.

Regulation (EC) No 152/2009 has laid down the methods of sampling and analysis for the official control of feed [10]. The new Regulation (EU) No 51/2013 includes new PCR-based methods for the detection of animal constituents [4]. Using qPCR methods it is possible to identify PAP samples because of the presence of remaining species specific intact DNA fragments in the samples. DNA based analyses are particularly applicable to the detection of these DNA fragments since they can still be detected in processed feed samples in which the target protein concentration would be below the limit of detection of protein-based assays.

A qPCR method for the detection of animal DNA in feed stuff has been validated by the EU Reference Laboratory (EURL-AP). Calibrants are needed to enable the implementation of this qPCR protocol used as an official control method for detecting the presence of PAPs in the feed. In particular, these calibrants are used to determine a threshold or cut-off value at which the PCR signal is considered to be negative thereby distinguishing positive from negative samples. The threshold value varies from one laboratory to another as qPCR quantification cycle (Cq) values are influenced by a large number of factors including the composition of the mastermix and the instrument used [11]. Therefore, each control laboratory must determine its own threshold value and verify this value on a regular basis.

To fulfil this objective, certified reference materials (CRMs) are required. The first CRM of this kind was produced for the detection of PAPs of ruminant origin (ERM-AD482). In the scope of the current project, JRC-IRMM has produced ERM-AD483 for the detection of PAPs of porcine origin. This CRM for porcine detection is required not only because of the reauthorized use of porcine PAPs in aquacultures since 2013 [9], but also because of the expectation that in 2015, the ban on PAPs in feed will be further lifted and porcine PAPs will be allowed in poultry feed.

1.2 Choice of the material

ERM-AD483a, b and c are certified for their absolute DNA copy number concentration and allow control laboratories to determine their cut-off values for the porcine assay [3, 11] and to report their results in line with European Commission Regulation (EU) No 51/2013 [4]. The plasmid of ERM-AD483a, b and c contains a porcine specific target DNA fragment of 83 base pairs that can be amplified using two specific primers. Both the target and the primers/probe sequences were designed and provided to IRMM by EURL-AP. The three calibrants are certified for their plasmid DNA copy number concentrations.

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 the 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 material has then been processed and DNA sequencing, homogeneity, characterisation, freeze-thaw, short- and long-term stability studies have been carried out by dPCR and ddPCR.

2. Participants

2.1 Starting material provider

European Union Reference Laboratory in the detection of animal proteins in feedingstuffs (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

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:

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

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

National Institute of Biology, Ljubljana, SI

TATAA Biocenter AB, Göteborg, SE

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

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

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

3. Material processing and process control

3.1 Origin and purity of the starting material

The pIRMM-0104 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, NL) 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-0104 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 may be present in the plasmid extract: a) plasmids used for the assembly of pIRMM-0104, 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: the plasmid of interest, and one of the plasmids used for the assembly of pIRMM-0104. The synthetic plasmids used for the cloning (pUC18 and pCR 2.1) have the same origin of replication (*oriV* from ColE1 plasmid) which allows them to replicate independently from the host chromosome (*E. coli*). 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 [12]. To ensure that the plasmid production started from a single bacterial clone, and would therefore contain only one type of plasmid, an additional plating step was included. A single colony containing the pIRMM-0104 was plated on an LB plate supplemented with ampicillin (50 µg/mL) and from this plate one isolated colony was picked for processing.

After enzymatic digestion with *SmaI* 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 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 linearized plasmid was analysed by spectrophotometry to detect the presence of non-nucleic acid contaminants e.g. proteins. The UV absorbance of the plasmid in solution was measured at 260 nm (A_{260}) and 280 nm (A_{280}), where pure nucleic acid samples are expected to have a theoretical A_{260}/A_{280} ratio of approximately 1.8 [13]. The A_{260}/A_{280} was measured as 1.83, indicating a sufficient DNA purity of the solution. However, this value does not exclude the presence of traces of contaminating proteins or chemical residues from nucleic acid extraction.

Contamination of the stock solution of plasmid pIRMM-0104 with traces of nucleic acids from 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. Nevertheless, 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 with other DNA fragments which could be present in the final plasmid preparation. Inconsistencies between the estimations of

the copy number concentration based on spectrophotometry and dPCR can, therefore, be used as an indication of the presence of contaminants in the plasmid solution.

Based on DNA mass concentration, the copy number concentration of the plasmid solution was estimated, applying equation 1:

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

c_{DNA} DNA mass concentration [ng/µl]

N_A Avogadro constant [cp/mol]

$M_{plasmid}$ molar mass of the pIRMM-0104 plasmid [ng/mol]

Calculation of the molar mass of the linear pIRMM-0104 plasmid was based on the DNA sequence of the plasmid and the molar mass of each nucleotide monophosphate [14].

Nanodrop spectrophotometry measured the plasmid mass concentration as 17.4 ng/µl which gave a calculated copy number concentration of 5.65 x 10^9 cp/µl.

3.2 Processing

The plasmid stock solution was diluted in 1 mmol/L TE_{low} (TRIS, 0.01 mmol/L EDTA at pH 8.0) containing 51 ng/µL salmon sperm genomic DNA (*Oncerhynchus keta*, Sigma Aldrich Cat. No.: D-1626) to reach the target concentration of 1280 cp/µL. From this intermediate solution, three independent dilutions were gravimetrically prepared to obtain 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 into 1200 sterile Axygen pre-labelled low binding vials under sterile and cooled conditions. Once filled, all vials were manually closed and were stored at -20 °C. The sets of calibrants were then prepared by assembling one vial of each concentration level, with the same identity number, into a plastic box and are stored back at -20 °C. At no point after the initial freezing were the vials allowed to thaw.

3.3 Process control

The final plasmid concentration was verified by dPCR from Fluidigm as described in Annex A. The copy number concentration was also verified by droplet digital PCR (ddPCR) using a QX100 droplet digital PCR system from Bio-Rad as described in Annex B. The measured copy number concentrations were in close agreement with the dPCR measurements (data not shown).

4. Homogeneity study

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

4.1 Between-unit homogeneity

The between unit homogeneity was evaluated for each concentration level of ERM-AD483. The measurements were performed under repeatability conditions on vials randomly taken from the entire batch and analysed in a randomised order.

Ten vials were selected using a random stratified sampling scheme covering the whole batch for the between-unit homogeneity test. Each selected vial of each concentration level was analysed 5 times independently by dPCR. The measurements of the randomised replicates were performed on 15 digital arrays. The measurements were performed under repeatability conditions in a randomised manner which allowed the separation of a potential analytical trend from a possible trend in the filling sequence.

The mean values and corresponding standard deviations for each vial of the dPCR homogeneity measurements are shown in Figure 1:

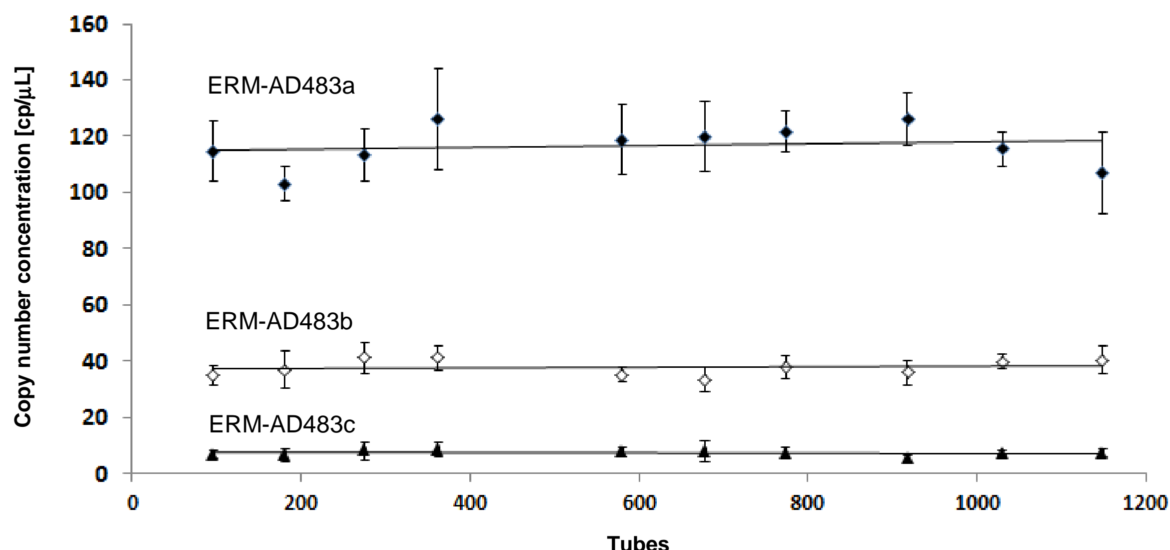

Figure 1: The 3 copy number concentrations measured by dPCR for level a (filled diamond), b (open diamond) and c (triangle) batches. Each point represents the average of 5 measurements. Error bars indicate the standard deviation at 95 % CI.

Regression analyses were performed to evaluate potential trends in the analytical sequence. Analytical trend was detected for ERM-AD483a and ERM-AD483b at 95 % CI, no analytical trend was observed for ERM-AD483c. Correction for this bias can improve the sensitivity of the subsequent statistical analysis through a reduction in analytical variation without masking potential between-unit heterogeneities. The analytical trend was corrected using the following formula:

Corrected result = Measured result $\times$ d_i **Equation 2**

With $d_i = \overline{x_1} / \overline{x_i}$

d_i	correction factor for plate i
$\overline{x_1}$	mean value measured for all vials on chip 1
$\overline{x_i}$	mean value measured for all vials on chip i (2 to 5)

The corrected dataset was then tested for trends in the filling sequence, which were not observed at a 95 % CI. The corrected dataset was then assessed for consistency using Single Grubbs outlier tests at a CI of 99 % both on the individual results and the unit means. No outliers were detected.

Between-unit inhomogeneity analysis was determined 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 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 distribution of the individual results is unimodal and of the unit means is normal.

It should be noted that $s_{bb,rel}$ and $s_{wb,rel}$ are estimates of the true standard deviations and, therefore, subject to random fluctuations. Therefore, the mean squares 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 4, 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 shown in Equation 5 and as described by Linsinger *et al.* [15]. 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.

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

$$s_{wb,rel} = \frac{\sqrt{MS_{within}}}{\overline{y}} \qquad \textbf{Equation 3}$$

$$s_{bb,rel} = \frac{\sqrt{\frac{MS_{between} - MS_{within}}{n}}}{\overline{y}} \qquad \textbf{Equation 4}$$

$$u^*_{bb,rel} = \frac{\sqrt{\frac{MS_{within}}{n}}\sqrt[4]{\frac{2}{\nu_{MSwithin}}}}{\overline{y}} \qquad \textbf{Equation 5}$$

MS_{within}	mean square within a unit from an ANOVA
$MS_{between}$	mean squares between-unit from an ANOVA
$\overline{y}$	mean of all results of the homogeneity study
n	mean number of replicates per unit
$\nu_{MSwithin}$	degrees of freedom of MS_{within}

The results of the evaluation of the within and between-unit variations are summarised in Table 1. The resulting values from the above equations were converted into relative uncertainties.

Since the homogeneity study showed no outlying unit means or trends in the filling sequence, the between-unit standard deviation (s_{bb}) can be used as the 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 1: Results of the between-unit homogeneity study, measured by dPCR

Material	n	$s_{wb,rel}$ [%]	$s_{bb,rel}$ [%]	$u^*_{bb,rel}$ [%]	$u_{bb,rel}$ [%]
ERM-AD483a	5	9.60	4.84	2.03	**4.84**
ERM-AD483b	5	11.72	5.53	2.48	**5.53**
ERM-AD483c	5	30.04	n.a.[1]	6.35	**6.35**

[1)] Cannot be calculated as $MS_{between} < MS_{within}$, n is the number of independent measurements.

4.2 Within-unit homogeneity and minimum sample intake

The within-unit homogeneity is closely correlated to the minimum sample intake. This correlation implies that 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. Thus, the 4 µL sample intake gives acceptable repeatability, demonstrating that the within-unit inhomogeneity does no longer contribute to analytical variation using this sample intake.

5. Stability study

Temperature, time and radiation are considered as the most relevant influences of the stability of a material. Hence, 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 [16] and under repeatability conditions which greatly improves the sensitivity of the stability tests. In this approach, samples were stored for specified length of time at different temperature conditions, whereupon they 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 a reference temperature of -70 °C. Samples were stored at two testing temperatures for safety reasons. Stability at -20 °C is tested to check the optimal transportation conditions and stability at +4 °C is tested to check the impact of elevated temperatures during transportation. Four units per storage time were selected using a random stratified sampling scheme. Each unit was measured once by dPCR and in three replicates by ddPCR. In case of the ddPCR measurements, not all the replicate measurements could be accepted due to technical reasons. The basis of technical rejection of a ddPCR measurement data is the unsatisfactory droplet quality. A measurement was accepted unless the number of generated droplets was below a threshold or when the so-called rain droplet profile was observed. 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 for each temperature on the pooled dPCR and ddPCR data set. The results were screened for outliers using the single and double Grubbs tests at 99 % CI. The results of the statistical evaluation of the short-term stability are summarised in Table 2.

Table 2: Uncertainty contribution estimated for 1 week of transportation by dPCR and ddPCR at -20 ºC and +4 ºC

Material	Number of individual outlying results at 99 % CI, dPCR and ddPCR		Significance of the trend at 95 % CI, dPCR and ddPCR		u_{sts} [%] dPCR and ddPCR at -20 °C	u_{sts} [%] dPCR and ddPCR at +4 °C
	-20 ºC	+4 ºC	-20 ºC	+4 ºC		
ERM-AD483a	none	none	no	no	**1.11**	**1.19**
ERM-AD483b	none	none	no	no	**1.06**	**1.37**
ERM-AD483c	none	none	no	no	**1.46**	**1.80**

No outliers were detected and none of the trends were statistically significant at 95 % CI neither at -20 °C nor at 4 °C and by none of the methods. Uncertainties were calculated at week 1, to cover the transportation time. Based on these data the material shall be shipped frozen on dry ice, however even at 4 °C it is stable enough. The short-term-stability profile obtained by both methods, i.e. dPCR and ddPCR are shown by Figure 2.

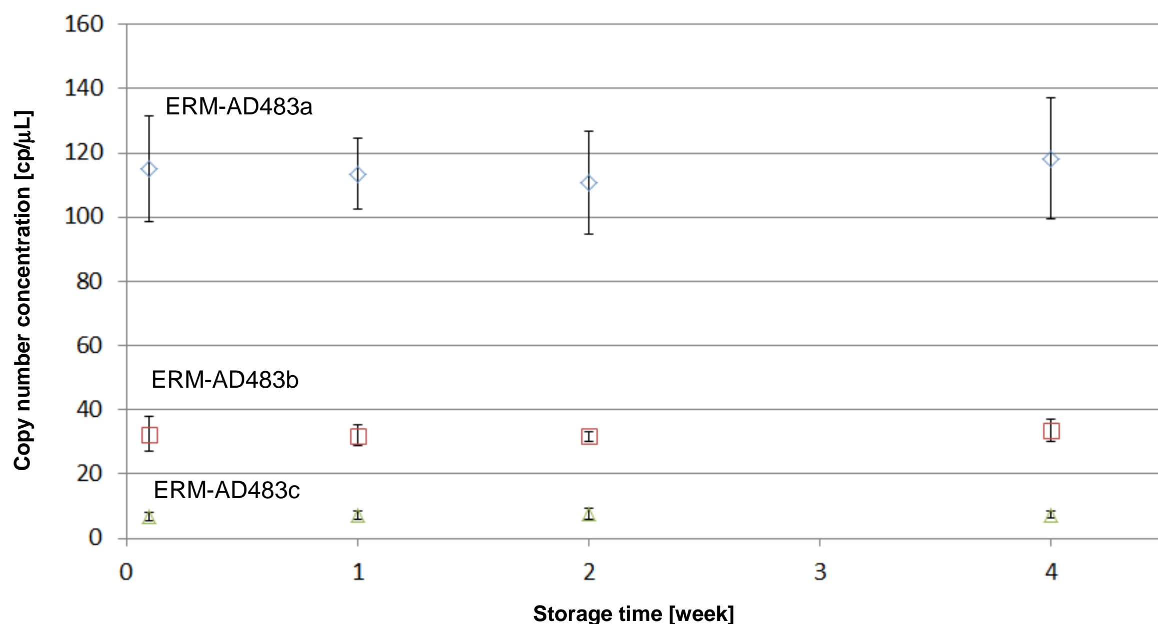

Figure 2: Stability of ERM-AD483a (blue diamond), b (red square) and c (green triangle) over 4 weeks. The error bars indicate standard deviations at CI of 95 %. The data shown are pooled data obtained by dPCR and ddPCR.

5.2 Long-term stability study

The long-term stability (LTS), i.e. storage conditions evaluation of ERM-AD483a, b and c was performed by storing the samples at -20 °C for each of 0, 2, 4, 6 and 12 months. The reference temperature was set to -70 °C. Five units per storage time were selected using a random stratified sampling scheme. Each unit was measured once using ddPCR, with the different concentration levels measured on different plates and on different days. The measurements were performed under repeatability conditions and in a randomised order to separate a potential analytical trend from a trend over storage time. Not all the replicate measurements could be accepted due to technical reasons. The basis of technical rejection of a ddPCR measurement data is the unsatisfactory droplet quality. A measurement was

accepted unless the number of generated droplets was below a threshold or when the so-called rain droplet profile was observed. The results were screened for outliers using the single and double Grubbs tests. No outliers were observed and none of the trends were statistically significant on a 99 % CI. The results of the statistical evaluation of the long-term stability are summarised in Table 3.

Table 3: Uncertainty contribution estimated for 1 year storage by ddPCR at -20 °C

Material	Number of individual outlying results, at 99 % CI	Significance of the trend at 95 % CI	u_{LTS} [%]
ERM-AD483a	none	no	**4.28**
ERM-AD483b	none	no	**4.92**
ERM-AD483c	none	no	**9.59**

The data were plotted against storage time (Figure 3) and regression lines of copy number concentration versus time were calculated. No significant regression was found (99 % CI). The uncertainty contribution of LTS was calculated at 12 months and the values are shown in Table 3.

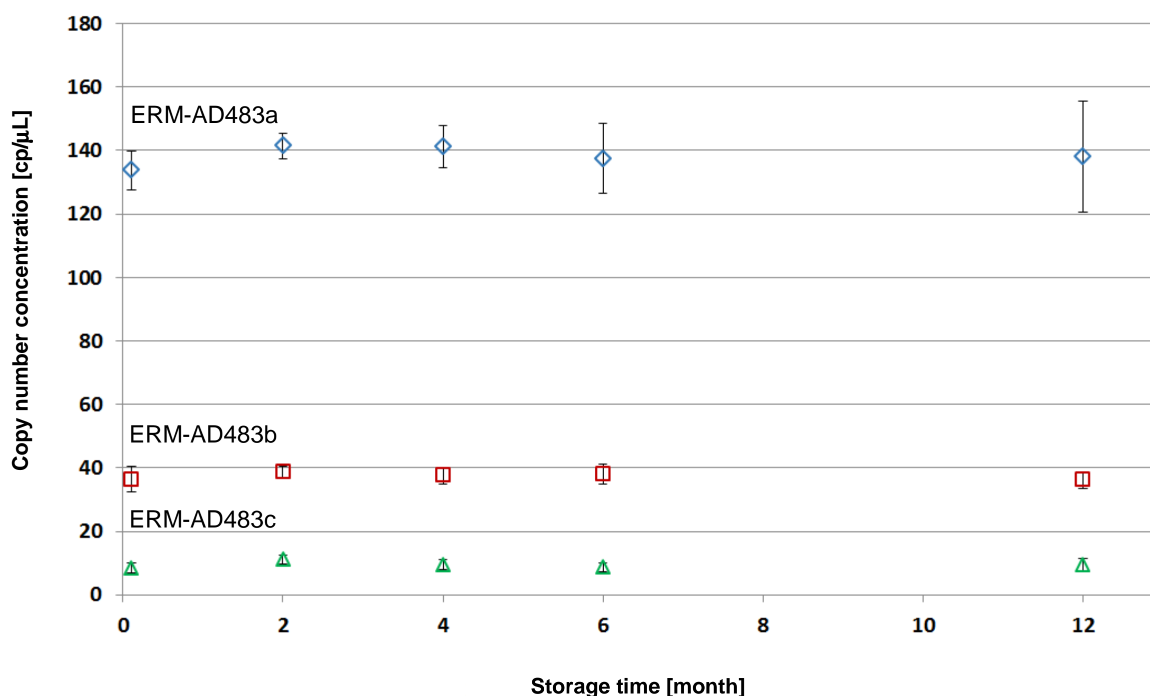

Figure 3: Changes in copy number of ERM-AD483a (blue diamond), b (red square) and c (green triangle), over 12 months. The solid red line indicates the certified values. The red dashed lines indicate the combined expanded uncertainty of the certified values. The error bars indicate standard deviations at CI of 95 %. The data shown are obtained by ddPCR.

5.3 Freeze-thaw study

A typical sample intake in a qPCR test is 5 µL and therefore 15 µL of each of the three calibration solutions are needed to establish a calibration curve with 3 replicates per concentration level. Each vial contains at least 1000 µL solution, which can be used to construct a minimum of 65 calibration curves per set. As the recommended storage temperature for the CRM set is -20 °C, repeated use of the CRM will require repeated freeze-thaw cycles. The effect of repeated freeze-thaw cycles was therefore investigated.

A similar approach to the isochronous design was followed in this study. Vials from ERM-AD483a, ERM-AD483b and ERM-AD483c were analysed. For each concentration, two vials were exposed to 0, 1, 2, 3, 4 and 5 freeze-thaw cycles and moved to the reference temperature (-20 °C) until analysis.

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

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 ERM-AD483b and ERM-AD483c, the slopes of the regression lines were not significantly different from zero (at 99 % CI). For the test vials of ERM-AD483a, the slope of the regression line was significantly different from zero (at 99 % CI), but measured values stayed within the combined expanded uncertainty boundaries (Figure 4). Based on these data, the CRMs should be aliquotted and none of the CRMs should be exposed to more than a total of 3 freeze-thaw cycles.

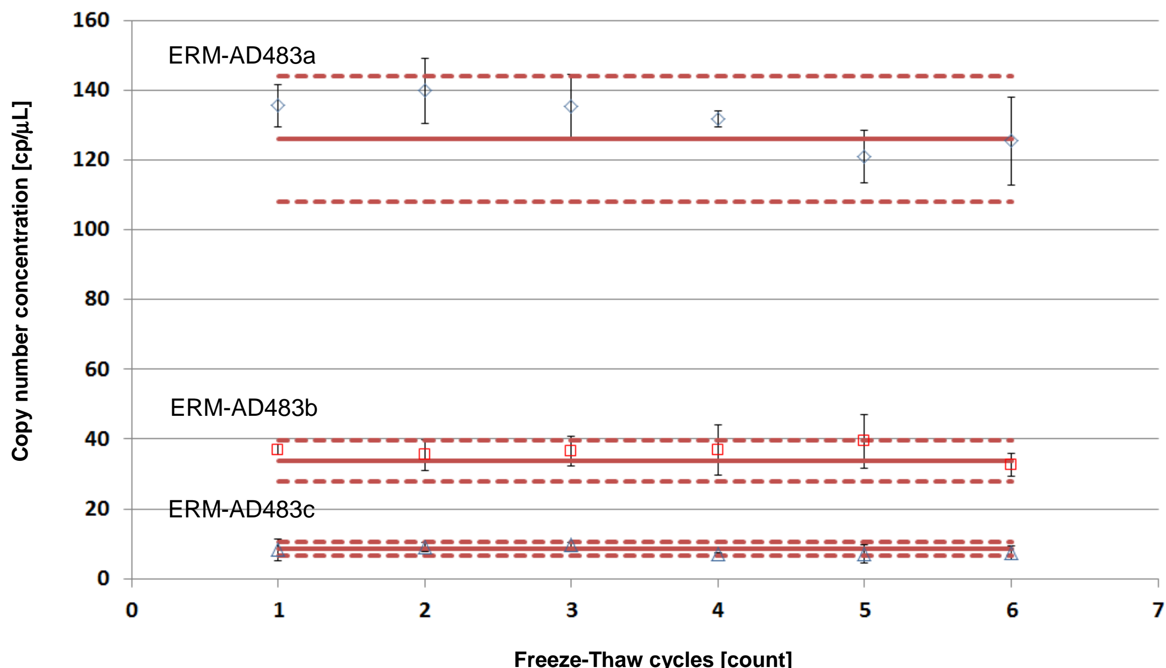

Figure 4: Freeze-thaw stability of ERM-AD483a (blue diamond), b (red square) and c (green triangle) over five cycles. The solid red line indicates the certified values. The red dashed lines indicate the combined expanded uncertainty of the certified values. The averages were calculated over $n = 2$. The error bars indicate standard deviations at CI of 95 %.

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 of the pIRMM-0104 sequence

The sequence of pIRMM-0104 was determined by dideoxy terminator sequencing (or Sanger sequencing) applying 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 a vial containing 30 µL of circular pIRMM-0104 plasmid dissolved in TE_{low} buffer, which had been set aside during the processing. The companies were commissioned to perform double stranded sequencing of an entire circular plasmid by primer walking method. Sequences were generated using the BigDye® Terminator chemistry version 3.0 (ABI). The sequence reactions were analysed on an ABI3730 capillary sequencer.

6.1.2 Results

Each company provided a full sequence of the pIRMM-0104 plasmid. Comparison of both sequences showed that they were identical to each other and were identical to the theoretical sequence, except for one nucleotide at the position 2236. This is a cytosine, as sequenced by the two companies (Seq1 and Seq2) and thymine in the theoretical sequence (Seq3), as shown by Annex C. Since Seq3 is an *in silico* sequence and because the mutation does not form part of the amplified sequence, which is indicated by yellow for the primer and cyan for the probe binding sites, it is, therefore, irrelevant. One of the companies also delivered the sequence quality values. The highest calculated cumulative error rate for the entire plasmid was found to be marginal: $1/1.4139 \times 10^9$.

6.2 Characterisation study for the copy number concentration of the plasmid

The material characterisation was based on an interlaboratory comparison study performed by expert laboratories. dPCR and ddPCR measurements determined the certified value for the three different copy number concentrations of the plasmid. The participants used the BioMark system (Fluidigm) with the 12.765 digital arrays to perform the dPCR measurements and the QX100 system to perform the ddPCR measurements. This study design results in the randomisation of laboratory bias, thereby reducing the combined uncertainty.

6.2.1 Study set up

Seven laboratories were selected based on criteria that comprised aspects of both technical competence and quality management. Each participant was required to operate a quality system and to deliver documented evidence of its laboratory proficiency in the field of digital

PCR measurements. Holding a formal accreditation was not mandatory, but meeting the requirements of ISO/IEC 17025 was obligatory.

dPCR measurements were performed by all the seven laboratories. Four out of the seven laboratories also performed ddPCR measurements. Each laboratory received two vials of each plasmid concentration of ERM-AD483 and was commissioned to provide two sets of results, one set per vial. Vials were selected using a random stratified sampling scheme covering the whole batch. None of the samples was diluted prior to the measurements. Each vial was analysed on five panels of one digital array. Each of the two vials was measured on different days and on different digital arrays or plates to ensure intermediate precision conditions were met. The PCR conditions for the characterisation study are shown in Annex A.

6.2.2 Results

The characterisation study resulted in seven independent dPCR and ddPCR pooled measurement results per CRM. The acquired data were first checked for compliance with the requested analysis protocol and their validity based on technical reasons. The following criteria were considered during the evaluation:

- compliance with the analysis protocol: sample preparations and measurements performed on 6 different days
- correctness of the reaction mix steps: all dilutions steps should have been prepared gravimetrically, and their masses were recorded
- a visual check to ascertain that no air bubble was present in the panels
- each independent set of dPCR measurement result should be based on the results of 5 panels
- ddPCR measurement results should be checked for droplet quantity and quality, i.e. the number of generated droplets should be more than 10000, and the droplets should not have a rain droplet profile

Based on the above criteria, all the seven measurement sets were retained from the laboratories.

The data were assessed for normality by using kurtosis/skewness tests and normal probability plots and were tested for outlying means using the Grubbs test and the Cochran test for outlying standard deviations (both at 99 % CI). The dataset means followed normal distributions and none of the data contained outlying means. The datasets were therefore consistent, and the means 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. with s the relative standard deviation of the technically valid independent measurements results and n the number of technically valid independent measurements. The results of these evaluations are shown in Table 4.

Table 4: Statistical evaluation for ERM-AD483, dPCR and ddPCR combined

Copy number concentration of the plasmid								
CRM	n	Outliers	Outliers	Statistical parameters				
		Means	Variances	Normally distributed	Mean [cp/μL]	s [cp/μL]	u_{char} [cp/μL]	$\boldsymbol{u_{char,rel}}$ **[%]**
ERM-AD483a	7	none	none	yes	125.99	8.02	3.03	**2.41**
ERM-AD483b	7	none	none	yes	33.78	3.55	1.34	**3.98**
ERM-AD483c	7	none	none	yes	8.53	1.29	0.49	**5.69**

Legends: n number of technically valid independent datasets, 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 require pooling of not less than 6 datasets to assign certified values. Full uncertainty budgets following the 'Guide to the Expression of Uncertainty in Measurement' [5] were established. ERM-AD483 was certified for its copy number concentration by dPCR and ddPCR.

7.1 Copy number concentration

The unweighted mean of the accepted datasets, as shown in Table 5, was assigned as certified value for the copy number concentration of the plasmid. The value of the copy number concentration of the plasmid is traceable to SI. Traceability of the obtained results is based on the traceability of all relevant input factors. Instruments in individual laboratories were calibrated with tools ensuring traceability to the SI. Consistency in the intercomparison 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.

The assigned uncertainty consists of uncertainties relating to characterisation, u_{char}, to potential degradation during long-term storage, u_{LTS}, to potential degradation during transportation, u_{STS}, and to potential between-unit heterogeneity, u_{bb}. These different contributions were combined to estimate the relative expanded uncertainty of the certified value ($U_{\mathrm{CRM,\,rel}}$) with a coverage factor k as:

$$U_{\mathrm{CRM,rel}} = k \cdot \sqrt{u_{\mathrm{char,rel}}^2 + u_{\mathrm{STS,rel}}^2 + u_{\mathrm{LTS,rel}}^2 + u_{\mathrm{bb,rel}}^2}$$ **Equation 6**

$u_{\mathrm{char,rel}}$ was estimated as described in Section 6

$u_{\mathrm{STS,rel}}$ was estimated as described in Section 5

$u_{\mathrm{LTS,rel}}$ was estimated as described in Section 5

$u_{\mathrm{bb,rel}}$ was estimated as described in Section 4

There were sufficient numbers of the degrees of freedom of the different uncertainty contributions, as measured by dPCR and ddPCR, and hence a coverage factor k of 2 was applied to calculate the expanded uncertainties.

The certified values and their uncertainties are summarised in Table 5.

Table 5: Certified values and their uncertainties for the copy number concentration of ERM-AD483

CRM	**Copy number concentration of the plasmid [cp/µL]**	$u_{char, rel}$ [%]	$u_{bb, rel}$ [%]	$u_{STS, rel}$ [%]	$u_{LTS, rel}$ [%]	$U_{CRM, rel}$ [%]	U_{CRM} [cp/µL]
	dPCR/ddPCR combined set	dPCR/ddPCR combined set	dPCR	dPCR/ddPCR combined set	ddPCR		
ERM-AD483a	**126**	2.41	4.84	1.11	4.28	**14**	**18**
ERM-AD483b	**34**	3.98	5.53	1.06	4.92	**17**	**6**
ERM-AD483c	**9**	5.69	6.35	1.46	9.59	**26**	**3**

8. Metrological traceability

8.1 Metrological traceability

8.1.1 Quantity value and identity of the measurand

The DNA copy number concentration of ERM-AD483 is a method-defined measurand and has been obtained by dPCR and ddPCR using primers, probe and PCR conditions shown in Annex A, Table A1 and A2. 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 plasmid DNA sequence and hence its identity was confirmed by two expert sequencing service companies using dye terminator cycle sequencing and 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 the absence of measurement bias. The identity of the measurand 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 and ddPCR.

9. Instructions for use

9.1 Safety information

The usual laboratory safety measures apply.

9.2 Storage conditions

The materials should be stored at -20 °C ± 5 °C prior to using. The plasmid containing solution should not be exposed to direct sunlight.

It is advisable to close the vial with the original screw cap after use. The solutions can be used for several experiments assuming that major contaminations during the handling of opened vials are excluded. The material should however not undergo more than 3 freeze-thaw cycles and therefore it is recommended to aliquot the solutions immediately at first use. Additionally, 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.

Please note that the European Commission cannot be held responsible for changes which may occur happen 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 for use, the contents of the vials must 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 designed to be used to determine the cut-off values by the Standard Operating Procedures, edited by the EURL-AP [3] for the technical implementation of Regulation (EC) No 152/2009 [10].

The minimum sample intake is 4 µL.

10. Acknowledgements

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11. Annex A: Description of the dPCR method

The protocol described here was used in all the measurements at IRMM, as well as by all the laboratories participating in the characterisation study. The primers and probes are those provided by the EURL-AP for the qPCR protocol. Volumes of 4 µL of the undiluted ERM-AD483a, ERM-AD483b, ERM-AD483c plasmid solutions were used 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 porcine target at final concentrations outlined in 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, which were 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 porcine target in a simplex dPCR reaction

PCR target	Primer/ probe	Sequence	Concentration in PCR reaction [nM]	Amplicon size [bp]
porcine target	Forward primer	5'-ACA ACA TAA TCT GAA TCA ATG C -3'	600	83
	Reverse primer	5'-TTC GCC TAG TTG GTT TAG TAG -3'	600	
	Probe	FAM- 5'-AGT ACA TAG TCT CCT CAT TAG CCT GAT C-3' TAMRA	250	

Table A2: Thermal cycling protocol used for the simplex dPCR reaction amplifying the porcine 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	50	

12. Annex B: Description of the ddPCR method

The protocol described here was used to all the measurements at IRMM, as well as by all the laboratories participating in the characterisation study. The primers and probes were those, which were used for dPCR and were provided by the EURL-AP for qPCR. Volumes of 7.7 µL of the undiluted ERM-AD483a, ERM-AD483b, ERM-AD483c plasmid solutions were used in the ddPCR assay. A volume of 7.7 µL of the DNA sample was mixed with 14.3 µL of Pre-sample mix solution and 20 µL of this mixture was loaded into a well of the droplet generator strip. 40 µL droplets were generated and loaded per well of a 96-well semi-skirted PCR plate. The 14.3 µL Pre-sample mix solution contains the primers and probes (1.1 µL each) for the porcine target at final concentrations as outlined in Table A1, together with 2x ddPCR supermix reagent (Cat. No.: 186-3010) (11 µL) from Bio-Rad (Bio-Rad Laboratories N.V., Temse, BE). The amplification was performed according to the specifications in Table A2. After amplification the droplets were read and the results analysed with the Quanta Soft V software using the appropriate threshold for each analysis.

13. Annex C: Sequence alignment of pIRMM-0104

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Seq1        GAGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGG 60
Seq2        GAGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGG 60
Seq3        GAGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGG 60
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Seq2        CACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAG 120
Seq3        CACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAG 120
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Seq2        CTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGA 180
Seq3        CTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGA 180
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Seq1        CAGCCTGAATGGCGAATGGCGCCTGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTAT 660
Seq2        CAGCCTGAATGGCGAATGGCGCCTGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTAT 660
Seq3        CAGCCTGAATGGCGAATGGCGCCTGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTAT 660
            ************************************************************

Seq1        TTCACACCGCATATGGTGCACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCA 720
Seq2        TTCACACCGCATATGGTGCACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCA 720
Seq3        TTCACACCGCATATGGTGCACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCA 720
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Seq1        GCCCCGACACCCGCCAACACCCGCTGACGCGCCCTGACGGGCTTGTCTGCTCCCGGCATC 780
Seq2        GCCCCGACACCCGCCAACACCCGCTGACGCGCCCTGACGGGCTTGTCTGCTCCCGGCATC 780
Seq3        GCCCCGACACCCGCCAACACCCGCTGACGCGCCCTGACGGGCTTGTCTGCTCCCGGCATC 780
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Seq1        CGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATGTGTCAGAGGTTTTCACCGTC 840
Seq2        CGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATGTGTCAGAGGTTTTCACCGTC 840
Seq3        CGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATGTGTCAGAGGTTTTCACCGTC 840
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Seq3        CCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACC 1020
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Seq3        CTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGT 1080
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Seq1        GTCAGACCAAGTTTACTCATATATACTTTAGATTGATTTAAAACTTCATTTTTAATTTAA 1980
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Seq1        AAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTT 2040
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Seq3        AAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTT 2040
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Seq1        TTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTT 2100
Seq2        TTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTT 2100
Seq3        TTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTT 2100
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Seq1        TTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTG 2160
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Seq3        TTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTG 2160
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Seq1        TTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCA 2220
Seq2        TTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCA 2220
Seq3        TTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCA 2220
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Seq1        GATACCAAATACTGTTCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGT 2280
Seq2        GATACCAAATACTGTTCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGT 2280
Seq3        GATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGT 2280
            *************** ********************************************

Seq1        AGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGA 2340
Seq2        AGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGA 2340
Seq3        AGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGA 2340
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Seq1        TAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTC 2400
Seq2        TAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTC 2400
Seq3        TAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTC 2400
            ************************************************************

Seq1        GGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACT 2460
Seq2        GGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACT 2460
Seq3        GGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACT 2460
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Seq1        GAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGA 2520
Seq2        GAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGA 2520
Seq3        GAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGA 2520
            ************************************************************

Seq1        CAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGG 2580
Seq2        CAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGG 2580
Seq3        CAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGG 2580
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Seq1        AAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATT 2640
Seq2        AAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATT 2640
Seq3        AAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATT 2640
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Seq1        TTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTT 2700
Seq2        TTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTT 2700
Seq3        TTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTT 2700
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Seq1        ACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGA 2760
Seq2        ACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGA 2760
Seq3        ACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGA 2760
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Seq1        TTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAAC 2820
Seq2        TTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAAC 2820
Seq3        TTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAAC 2820
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Seq1        GACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAA 2857
Seq2        GACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAA 2857
Seq3        GACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAA 2857
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Legend: Seq1 is provided by Eurofins, Seq2 is provided by BaseClear, and Seq3 is an *in silico* sequence theoretical sequence.

14. Annex D: Raw data of the characterisation study

Table A3: Raw data of the combined dPCR and ddPCR characterisation dataset of ERM-AD483a

Labs	Mean	s	CI 95 %	Samp#1 dPCR	Samp#2 dPCR	Samp#3 dPCR	Samp#4 dPCR	Samp#5 dPCR	Samp#6 dPCR	Samp#7 dPCR
L0-Coordinator	129.1271	14.3598	8.2911	156.32	140.84	136.98	148.85	138.23	122.59	114.68
L1-Lab 1	128.1893	9.4479	5.455	119	128.67	125.22	125.02	148.03	123.3	122.04
L2-Lab 2	128.577	7.7209	5.5232	139.77	136.27	133.92	136.36	119.47	121.18	120.33
L3-Lab 3	115.404	11.5905	8.2913	128.67	98.45	98.66	114.79	113.91	124.78	105.81
L4-Lab 4	138.8177	11.1423	6.7332	142.65	146.05	140.77	143.32	145.27	153.96	131.66
L5-Lab 5	116.6314	15.1051	8.7214	106.24	98.95	99.41	93.77	103.94	136.13	127.46
L6-Lab 6	125.173	8.7526	6.2613	118.29	136.44	130.02	125	120.42	112.74	122.67

Labs	Samp#8 dPCR	Samp#9 dPCR	Samp#10 dPCR	Samp#11 ddPCR	Samp#12 ddPCR	Samp#13 ddPCR	Samp#14 ddPCR
L0-Coordinator	119.73	108.2	109.01	132	120.91	132.13	127.31
L1-Lab 1	111.36	142.02	136.76	130.89	132.62	123.37	126.35
L2-Lab 2	123.61	131.3	123.56	-	-	-	-
L3-Lab 3	127.54	127	114.43	-	-	-	-
L4-Lab 4	-	152.66	147.94	130	121.7	126.66	121.99
L5-Lab 5	132.48	143.92	123.19	115.14	113.46	118.49	120.26
L6-Lab 6	137.7	132.87	115.58	-	-	-	-

Figure A1: Lab means and their standard deviation for the characterisation study of ERM-AD483a, dPCR and ddPCR combined dataset

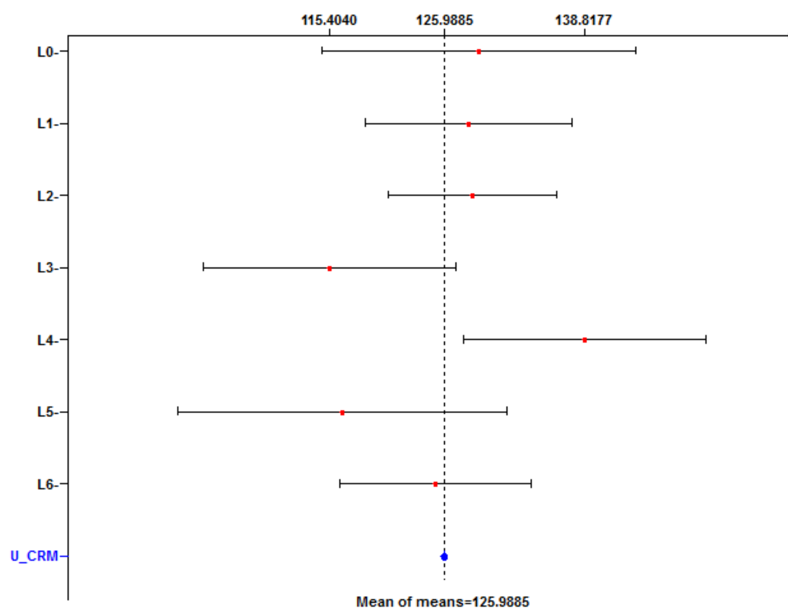


Table A4: Raw data of the combined dPCR and ddPCR characterisation dataset of ERM-AD483b

Labs	Mean	s	CI 95 %	Samp#1 dPCR	Samp#2 dPCR	Samp#3 dPCR	Samp#4 dPCR	Samp#5 dPCR	Samp#6 dPCR	Samp#7 dPCR
L0-Coordinator	32.4243	6.3837	3.6858	42.44	41.65	33.12	36.4	26.39	29.31	36
L1-Lab 1	33.36	2.4927	1.4392	29.91	27.04	36.4	32.59	32.65	34.39	32.71
L2-Lab 2	36.836	3.3478	2.3949	37.98	35.28	33.41	35.54	34.78	34.67	38.14
L3-Lab 3	32.49	4.7171	3.3744	37.61	35.55	32.8	30.3	25.35	24.8	34.91
L4-Lab 4	39.39	6.1875	3.5726	42.81	47.19	41.05	29.43	34.09	43.81	37.1
L5-Lab 5	28.1871	3.7503	2.1654	31.68	29.79	21.69	26.75	29.77	24.61	29.87
L6-Lab 6	33.793	8.0505	5.759	38.58	39.21	43.88	47.93	26.28	25.13	26.99

Labs	Samp#8 dPCR	Samp#9 dPCR	Samp#10 dPCR	Samp#11 ddPCR	Samp#12 ddPCR	Samp#13 ddPCR	Samp#14 ddPCR
L0-Coordinator	20.84	24.35	25.99	34.09	37.41	33.23	32.72
L1-Lab 1	33.31	35.86	33.39	34.25	36.22	33.61	34.71
L2-Lab 2	42.03	42.85	33.68	-	-	-	-
L3-Lab 3	30.41	34.86	38.31	-	-	-	-
L4-Lab 4	42.21	52.28	42.01	35.76	36.62	33.15	33.95
L5-Lab 5	23.76	21.71	30.09	31.58	32.4	29.79	31.13
L6-Lab 6	30.04	27.95	31.94	-	-	-	-

Figure A2: Lab means and their standard deviation for the characterisation study of ERM-AD483b, dPCR and ddPCR combined dataset

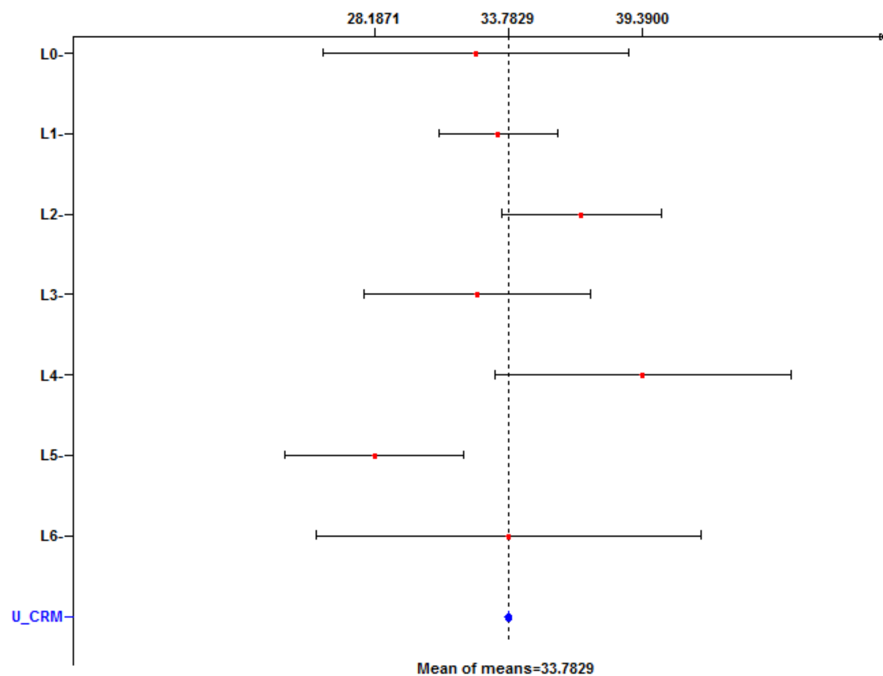
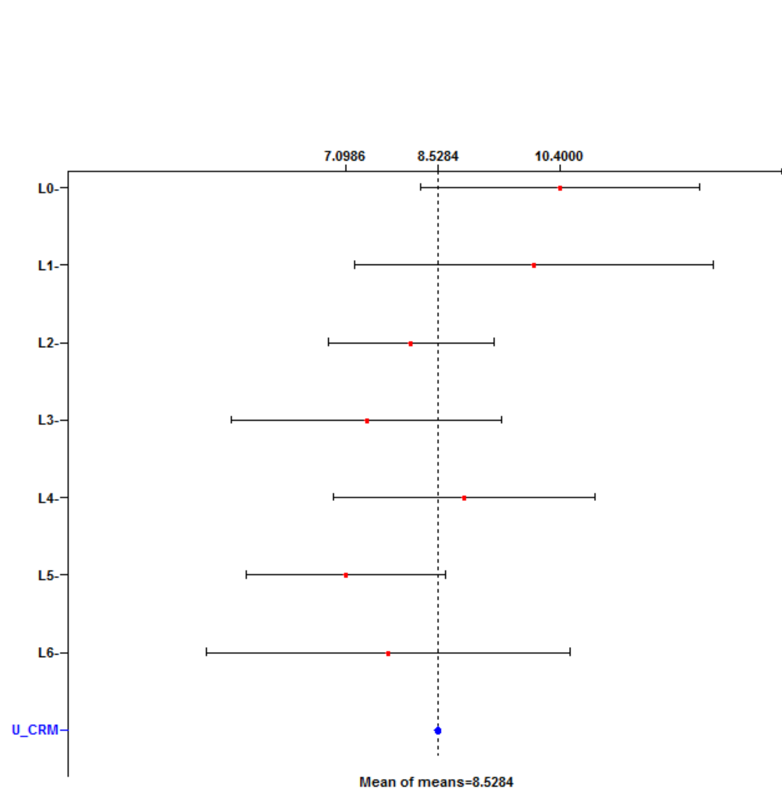


Table A5: Raw data of the combined dPCR and ddPCR characterisation dataset of ERM-AD483c

Labs	Mean	s	CI 95 %	Samp#1 dPCR	Samp#2 dPCR	Samp#3 dPCR	Samp#4 dPCR	Samp#5 dPCR	Samp#6 dPCR	Samp#7 dPCR
L0-Coordinator	10.4	2.151	1.2419	12.47	8.67	12.07	11.37	14.75	12.48	10.45
L1-Lab 1	10.0057	2.7696	1.5991	14.59	8.14	10.5	13.93	7.22	11.26	12.53
L2-Lab 2	8.105	1.2782	0.9144	9.16	6.52	6.62	7.93	7.42	7.14	10.66
L3-Lab 3	7.419	2.0761	1.4851	4.39	8.81	11.1	9.95	5.95	6.15	7.86
L4-Lab 4	8.9229	2.0117	1.1615	9.47	9.41	6.05	14.03	9.93	11.49	8.14
L5-Lab 5	7.0986	1.5364	0.8871	9.94	5.8	6.11	7.37	7.14	5.29	9.53
L6-Lab 6	7.748	2.8047	2.0064	12.84	11.24	5.97	5.58	7.71	3.37	7.07

Labs	Samp#8 dPCR	Samp#9 dPCR	Samp#10 dPCR	Samp#11 ddPCR	Samp#12 ddPCR	Samp#13 ddPCR	Samp#14 ddPCR
L0-Coordinator	11.44	10.83	8.1	8.22	7.59	8.75	8.41
L1-Lab 1	8.08	13.75	8.58	8.54	5.97	7.75	9.24
L2-Lab 2	8.22	9.02	8.36	-	-	-	-
L3-Lab 3	7.15	7.33	5.5	-	-	-	-
L4-Lab 4	8.85	7.12	6.62	8.48	8.65	8.27	8.41
L5-Lab 5	4.93	5.41	8.35	7.68	6.57	8.07	7.19
L6-Lab 6	9.36	6.19	8.15	-	-	-	-

Figure A3: Lab means and their standard deviation for the characterisation study of ERM-AD483c, dPCR and ddPCR combined dataset



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European Commission

EUR 27505 EN – Joint Research Centre – Institute for Reference Materials and Measurements

Title: **CERTIFICATION REPORT A set of three plasmid DNA calibration solutions bearing a porcine-specific DNA fragment**

Certified Reference Materials: ERM®-AD483a, ERM®-AD483b, ERM®-AD483c

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

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