European Commission

JRC REFERENCE MATERIALS REPORT

Certification of plasmid DNA copy number concentrations bearing a ruminant-specific DNA fragment in three buffered solutions: ERM®-AD482ka, ERM®-AD482kb, ERM®-AD482kc

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Abstract

ERM®-AD482k is a set of three reference materials produced within the scope of ISO 17034 [1] accreditation.

The European Union Reference Laboratory for Animal proteins in feedingstuffs inserted a ruminant - specific sequence into the pUC18 plasmid which was cloned into *E. coli*. The plasmid was multiplied, purified, linearised, quantified by UV spectrophotometry, sequenced by Sanger sequencing, and diluted gravimetrically to three target concentrations. Copy numbers were certified by digital PCR. The CRM consists of three polypropylene vials containing at least 1 mL of solution at one of the three certified concentrations.

Between-unit homogeneity was quantified and stability during transport and storage was assessed in accordance with ISO 33405:2024 [2].

The material was characterised by an interlaboratory comparison of laboratories of demonstrated competence and adhering to ISO/IEC 17025:2017 [3]. Technically invalid results were removed but no outlier was eliminated unless a technical reason for the deviation was found.

Uncertainties of the certified values include uncertainties related to possible inhomogeneity, instability and characterisation.

The material is intended for quantitative PCR, to determine a PCR platform's cut-off value, differentiating positive samples (containing the ruminant target sequence) from negative samples.

Before release of the three CRMs, the certification project was subjected to peer-review involving both internal and external experts.

Acknowledgements

The authors would like to acknowledge the support received from colleagues of JRC for the processing, organising of stability studies, measuring, reviewing of the certification project and distribution of this CRM.

Furthermore, the authors would like to thank the external experts of the Reference Material Review Panel Gilbert Berben (Centre Wallon De Recherches Agronomiques, Gembloux, BE) and Sofie Derycke (Instituut voor Landbouw- en Visserijonderzoek, Oostende, BE) for their constructive comments and reviews.

In addition, the authors would like to thank particularly, O. Fumière and A. Marien (European Union Reference Laboratory for Animal proteins in feedingstuffs (EURL-AP), Valorisation of Agricultural Products Department, Gembloux, BE) for having actively contributed to this project.

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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 aquaculture is regulated within the European Union through several regulations (Regulations of the European Parliament and of the Council (EC) 999/2001 and (EC) 1774/2002 and Commission Regulation (EC) 1234/2003) banning all PAPs from feed and banning intra-species recycling [4], [5] and [6]. In 2013, the ban of non-ruminant PAPs to be used in aquacultures was lifted [7] and in 2021, the ban of non-ruminant PAPs to be used in the feed of non-ruminant animals was lifted through Commission Regulation (EU) 2021/1372, maintaining the ban on intra-species recycling [8].

Following these Regulations, methods for species-specific detection of PAPs in compound feeds are required. Commission Regulation (EC) No 152/2009 lays down the methods of sampling and analysis for the official control of feed [9], lastly amended by Commission Implementing Regulation (EU) No 2020/1560 [10]. Quantitative polymerase chain reaction (qPCR) methods [11], allow to identify PAP samples through the presence of remaining species-specific, intact DNA fragments in the samples. DNA-based analyses are particularly suitable as the targeted DNA fragments can still be detected in processed feed samples in which the target protein concentration would be below the limit of detection (LOD) of protein-based assays.

Quantitative PCR methods for detecting animal DNA in feedstuffs have been validated by the European Union Reference Laboratory for Animal proteins in feedingstuffs (EURL-AP). To enable the implementation of these qPCR protocols as an official control method for detecting the presence of PAPs in feed, appropriate calibrants are required. These calibrants are used to establish the cut-off value, below which the qPCR signal is considered negative, thereby enabling the distinction between positive and negative samples. The cut-off value can vary between PCR platforms, as qPCR quantification cycle (Cq) values are influenced by multiple factors, including the composition of the master mix and the type of instrument used [12]. Therefore, each control laboratory must determine its own instrument- and method-specific threshold value and regularly verify it.

Therefore, certified reference materials (CRMs) are required. JRC has produced CRMs for the detection of PAPs of ruminant (ERM-AD482), porcine (ERM-AD483) and poultry (ERM-AD484k). As the ERM-AD482 needed to be replaced, a set of three new CRMs (ERM-AD482k) has been produced and made available.

1.2. Choice of the material

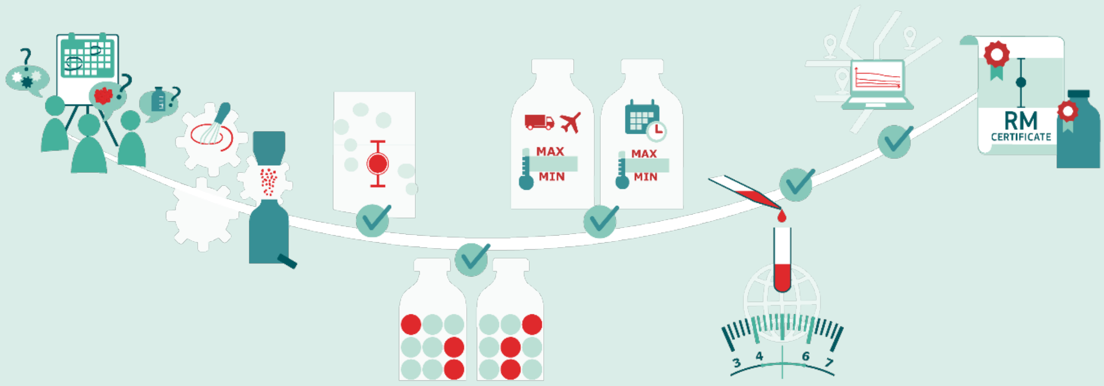
ERM-AD482k, with concentration levels a, b and c, consist of linearised plasmid DNA in buffered solution certified for their absolute DNA copy number concentration, expressed in copy number per microlitre (cp/µL). The CRMs allow control laboratories to determine the cut-off values of their ruminant assay [4, 12] and to report the results in line with Commission Regulation (EU) No 51/2013 [13].

The plasmid of ERM-AD482k contains a ruminant-specific nucleus target DNA fragment of 86 base pairs (bp) that can be amplified using two specific primers. Both the target and the primers/probe sequences were designed and provided to JRC by the EURL-AP.

The plasmid is stabilised using *Zea Mays* DNA as background DNA in the buffer. The material is made available as a liquid frozen material. The target concentration levels are set as defined by the standard operating procedure for the detection of ruminant DNA in feed using qPCR [11], validated by the EURL-AP.

1.3. Outline of the CRM project

Box 1. Reference material production



Reference material (RM) production is defined in ISO 17034 [1] as a project comprising planning and processing of the material, followed by homogeneity and stability testing, characterisation and assigning of one or more property values. Depending on the intended use of the RM a commutability study is carried out.

For certified reference materials (CRMs) a certificate is issued while for RMs a product information sheet is issued by the reference material producer (RMP).

CRMs and RMs are distributed globally and the stability of their assigned values is monitored throughout the life-time of the material.

An *Escherichia coli* (*E. coli*) strain containing a pUC18 plasmid modified with the DNA target of interest was provided by the EURL-AP. At the JRC, the plasmid DNA was multiplied, purified and linearised. The plasmid DNA concentration of the stock solution was assessed by UV spectrophotometry and measured more precisely by digital droplet PCR. The solution was diluted gravimetrically to obtain three solutions at target concentrations of 8 cp/µL, 32 cp/µL and 128 cp/µL, which were dispensed in polypropylene tubes. The three solutions were processed independently, beginning with the lowest concentration and progressing to the highest concentration.

Homogeneity and stability, including freeze-thaw stability, were assessed by droplet digital PCR (ddPCR).

Confirmation of the identity was done by Sanger sequencing.

The characterisation of the copy number concentration was assessed by an interlaboratory comparison involving several types of digital PCRs (chamber digital PCR (cdPCR), droplet digital PCR (ddPCR) and crystal droplet digital PCR (cddPCR).

Analogous to the independent processing conducted for the three materials, the scientific and statistical results of ERM-AD482ka, ERM-AD482kb and ERM-AD482kc were assessed independently.

Uncertainties of certified values were estimated in compliance with ISO 17034 [1], which implements the basic principles of ISO/IEC Guide 98 (GUM) [14].

The CRM project, including the certification approach and the evaluation of the obtained measurement data, was subjected to peer-review involving both internal and external experts.

Certain commercial equipment, instruments, and materials are identified in this report 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.

2 Participants

2.1 Raw material provider and development of the quantification method

Walloon Agricultural Research Centre (CRA-W), EURL-AP, Gembloux, BE

2.2 Project management and data evaluation

European Commission, Joint Research Centre, Directorate F – Health and Food, Geel, BE

(accredited to ISO 17034:2016 for production of certified reference materials, BELAC No. 268-RM)

2.3 Processing

European Commission, Joint Research Centre, Directorate F – Health and Food, Geel, BE

2.4 Homogeneity measurements

European Commission, Joint Research Centre, Directorate F – Health and Food, Geel, BE

2.5 Stability measurements

European Commission, Joint Research Centre, Directorate F – Health and Food, Geel, BE

2.6 Characterisation measurements

European Commission, Joint Research Centre, Directorate F – Health and Food, Geel, BE

National Institute of Biology, Ljubljana, SI

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

TATAA Biocenter AB Göteborg, Göteborg, SE

Walloon Agricultural Research Centre (CRA-W), EURL-AP, Gembloux, BE

All datasets are identified by a code (e.g. D01). The numbering is not related to the order of the laboratories presented above.

3 Material processing and processing control

Box 2. Reference material processing

RM processing covers the raw material conversion into a homogenous and stable material. It typically includes processing steps such as grinding or sieving and drying steps to enhance stability. When the processed material fulfils the specifications, the final material is filled into individual containers, referred to as RM units, such as bottles or ampoules and is labelled.

3.1 Origin and purity of the starting material

3.1.1 Origin of the plasmid

Provided in 2013 as a bacterial culture in Luria Bertani (LB) medium, supplemented with glycerol and stored at -70 °C, the plasmid of interest was constructed by cloning a ruminant specific nuclear DNA fragment of 86 bp into a pUC18 vector by the EURL-AP. The clone used for the production of the ERM-AD482 and ERM-AD482k was stored at -70 °C and recorded under the reference C127 of the internal collection database.

In 2022, the C127 bacterial culture was plated on the selective LB-agar medium supplemented with ampicillin. From this initial culture, two isolated colony forming units were chosen and cultivated further into 10 mL of liquid selective LB medium supplemented with ampicillin. The plasmid was extracted from these solutions and purified on a silica column (Qiaquick Gel extraction Mini kit, Qiagen, Venlo, NL) and suspended in sterile TE buffer (1 mM Tris, 0.01 mM EDTA, pH 8).

3.1.2 Plasmid conformation

In order to get blunt ends, the extracted plasmid was linearised by enzymatic digestion with restriction enzyme *Ssp*I. The digest was purified using silica columns (Qiaquick® Gel extraction kit, Qiagen, Venlo, NL), suspended in sterile TE buffer and kept at 4 °C before processing. The completeness of the enzymatic digestion and the size of the plasmid were confirmed by agarose gel electrophoresis (data not shown).

3.1.3 Plasmid identity confirmation

The identity of the plasmid was confirmed by sequencing and comparison of the obtained sequence with the reference sequence provided by the EURL-AP.

The sequence of the plasmid was determined by Sanger sequencing of the entire circular plasmid, applying the primer walking method. The Sanger sequencing technique is based on the selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase during *in vitro* DNA replication.

Sequencing was performed by Eurofins BV (Leiden, NL). The selection of the company was based on criteria that comprised aspects of both technical competence and quality management aspects. The company was required to operate a quality system and to deliver documented evidence of its laboratory proficiency in the field of sequencing. Holding a formal accreditation was not mandatory, but meeting the requirements of ISO/IEC 17025 [3] was obligatory.

The company received a sample of the circular plasmid solution, which had been set aside before processing and performed double stranded sequencing of the entire circular plasmid by applying the Sanger sequencing method. Sequences were generated using the BigDye® v3.1 chemistry from Thermo Fisher Scientific. The sequence reactions were analysed on an ABI3730xl sequencer from Thermo Fisher Scientific. A full sequence of the plasmid was obtained and compared with the reference sequence provided by the EURL-AP.

Sequences were aligned using the Basic Local Alignment search Tool (BLAST) [15]. Alignment confirmed a 99.94 % match between the sequences with one missing nucleotide (position 431) and one single mismatch (position 2237) between the sequences. The missing nucleotide is located in the insert but does not affect the amplification of the fragment of interest for ruminant PCR assays. The single mismatch is located in the fragment originating from the pUC18 vector and has therefore no effect on the suitability of the reference material. The result of the alignment can be found in Annex 1.

3.2 Processing

3.2.1 Determination of the plasmid concentration

3.2.1.1 Estimation of plasmid concentration by spectrophotometry

The linearised 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 the wavelengths of 260 nm (A260) and 280 nm (A280), where pure nucleic acid samples have a theoretical A260/A280 ratio of approximately 1.80 [16]. The A260/A280 ratio was measured as 1.79, 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 purified plasmid solution with traces of nucleic acids from the host bacterial cell proteins may affect the DNA mass concentration measured by spectrophotometry. 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 PCR measurements, as the primers and probes are highly specific for the targeted sequences and do not hybridise with other DNA fragments.

Based on DNA mass concentration, the copy number concentration of the plasmid solution was estimated, as shown below:

$$copy\ number\ concentration = \frac{c_{DNA} \cdot \mathrm{N}_A}{\mathrm{M}_{plasmid}} \qquad \textbf{Equation 1}$$

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

N_{A} Avogadro constant [cp/mol]

$\mathrm{M}_{plasmid}$ molar mass of the plasmid [ng/mol]

The Avogadro constant represents in this specific case the number of copies of plasmid in one mole equal to 6.023×10^{23} copies per mole.

Calculation of the molar mass of the linear plasmid was based on the length of the plasmid (2860 bp) and on the molar mass (615 g/mol) of each nucleotide monophosphate [17].

Nanodrop spectrophotometry was used to measure the plasmid mass concentration of the stock solution, which was determined to be 14.6 ng/µL. This corresponds to a copy number concentration of 5.0×10^{9} cp/µL. Using this value, the necessary dilutions were calculated to prepare a 500 mL mother stock solution containing approximately 1500 cp/µL of plasmids. The plasmid solutions were diluted using a sterile TE buffer supplemented with a maize DNA adjusted at a final concentration 12 ng/µL.

3.2.1.2 Confirmation of plasmid concentration by ddPCR

The copy number concentration of the plasmid in the mother stock solution was subsequently verified by ddPCR, which gave a measured value of approximately 700 cp/µL. This concentration in the stock solution was considered as insufficient to produce the desired, ERM-AD482ka, ERM-AD482kb, and ERM-AD482kc materials.

To address this, the mother stock solution was spiked with 7.5 mL of a solution from the previous dilution step, which had been set to 5×10^{4} cp/µL. After spiking, the concentration of the mother stock solution was re-evaluated using ddPCR, yielding a final concentration and corresponding standard deviation of (1493 ± 40) cp/µL. The mother stock solution was kept at 4 °C until further processing.

3.2.1.3 Production of the daughter stock solutions

From the mother stock solution, three independent dilutions with a sterile TE buffer containing 12 ng/µL of maize DNA were gravimetrically prepared to obtain approximately 2 L of daughter solutions, each with the following target concentrations: 8 cp/µL, 32 cp/µL and 128 cp/µL.

3.2.1.4 *Filling and packaging*

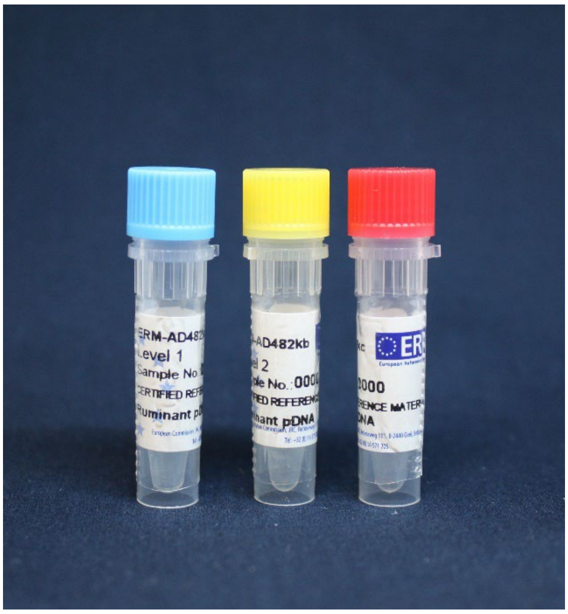
From each daughter solution, a volume of 1.1 mL was dispensed into 1800 sterile pre-labelled, low binding polypropylene vials (Axygen, Mexico City, MX; cat. No. SCT-150-SS-L-S) under sterile and cooled conditions. Once filled, a random stratified sampling scheme was applied at that stage and all vials were finally stored at -20 °C.

Sets of the plasmid solutions were prepared by assembling one vial of each concentration level into a box. After the initial freezing the vials were not allowed to thaw.

For the purpose of this report, the term 'unit' refers to one vial of ERM-AD482ka, ERM-AD482kb or ERM-AD482kc.

One unit of each concentration level of ERM-AD482k is shown in Figure 1.

Figure 1. Units of ERM-AD482ka, ERM-AD482kb and ERM-AD482kc.



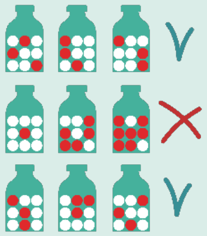
Source: JRC

3.3 Processing control

The plasmid copy number concentrations in the ERM-AD482kc, ERM-AD482kb and ERM-AD482ka were verified by ddPCR on the day of processing. The measured copy number concentrations were in agreement with the target concentrations (data not shown).

4 Homogeneity

Box 3. Homogeneity assessment



A key requirement for any RM produced as a batch of units is equivalence between those units. It is important to know how much the variation between units contributes to the uncertainty of the certified value. Consequently, ISO 17034 [1] requires RMPs to quantify the between-unit variation in homogeneity studies.

The within-unit homogeneity is correlated to the minimum sample size, which is the minimum amount of sample that is, for a given measurand, representative of the whole unit and that should be used in an analysis. Using sample intakes equal to or above the minimum sample size guarantees the assigned value within its stated uncertainty.

The within-unit inhomogeneity does not influence the uncertainty of the certified value when the minimum sample size is respected, but determines the minimum size of sample that is representative for the whole unit. Quantification of within-unit homogeneity is therefore necessary to determine the minimum sample size.

4.1 Between-unit homogeneity

The between-unit homogeneity of each material ERM-AD482kc, ERM-AD482kb and ERM-AD482ka was evaluated to ensure that the certified values of the CRMs are valid for all units of the material, within the stated uncertainties.

The number of units selected corresponds to approximately the cube root of the total number of units produced. A random stratified sampling scheme was used to select 14 units for the between-unit homogeneity test, covering the entire batch of each material. The batch was divided into 14 strata, each containing a similar number of units, and one unit was randomly selected from each stratum. From each selected unit, a unique sample was prepared and mixed with the master mix solution. Six replicates of each sample were then analysed by ddPCR. The measurements were performed under repeatability conditions and in a randomised manner to separate a potential drift in the measurement results from a potential trend in the filling sequence. The results are shown as graphs in Annex 2.

Regression analyses were performed to evaluate potential trends in the measurement sequence as well as trends in the filling sequence. No trends in the filling sequence or the measurement sequence were observed at a 95 % confidence level.

Quantification of between-unit inhomogeneity of each material (ERM-AD482kc, ERM-AD482kb and ERM- AD482ka) was undertaken by analysis of variance (ANOVA), which separates 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 were representative for the whole unit.

Evaluation by ANOVA requires mean values per unit that follow at least a unimodal distribution and results for each unit that follow unimodal distributions with approximately the same standard deviations. The distribution of the mean values per unit was visually tested using histograms and normal probability plots. Minor deviations from unimodality of the individual values do not significantly affect the estimate of between-unit standard deviations. The results of all statistical evaluations are given in Table 1.

Table 1. Results of the statistical evaluation of the homogeneity studies.

CRM-code	Trends [1)]		Outliers [2)]		Distribution	
	Measurement sequence	Filling sequence	Individual results	Unit means	Individual results	Unit means
ERM-AD482ka	no	no	none	none	normal	unimodal
ERM-AD482kb	no	no	none	none	normal	unimodal
ERM-AD482kc	no	no	none	none	normal	unimodal

1) 95 % confidence level.
2) 99 % confidence level.

Source: JRC

It should be noted that $s_{bb,\,rel}$ and $s_{wb,\,rel}$ are estimates of the standard deviations and are 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 a negative number under the square root used for the estimation of the between-unit variation, whereas the true variation cannot be less than zero. In this case, u^*_{bb}, the maximum inhomogeneity that could be hidden by method repeatability, was calculated as described by Linsinger *et al.* [18]. The uncertainty u^*_{bb} is comparable to the (LOD) of a measurement method yielding the maximum degree of inhomogeneity that might be undetected by the given study setup.

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

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

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

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

MS_{within} mean of squares within-unit from ANOVA
$MS_{between}$ mean of squares between-unit from ANOVA
$\bar{y}$ mean of all results of the homogeneity study
n mean number of replicate analysis per unit
$\nu_{MSwithin}$ degrees of freedom of MS_{within}

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 a relative uncertainties.

Table 2. Results of the homogeneity studies.

CRM-code	$s_{wb, rel}$ [%]	$s_{bb, rel}$ [%]	$u^*_{bb, rel}$ [%]	$u_{bb, rel}$ [%]
ERM-AD482ka	3.97	1.44	0.67	1.44
ERM-AD482kb	8.50	2.53	1.43	2.53
ERM-AD482kc	14.32	5.00	2.40	5.00

Source: JRC

The homogeneity studies showed no outlying unit means or trends in the filling sequence. As u^*_{bb} sets the limits of the study to detect inhomogeneity, the larger value of s_{bb} and u^*_{bb} is usually adopted as uncertainty contribution to account for potential inhomogeneity. Moreover, as outlined in ISO 33405:2024 [2], the experimental design used in this study allows the use of the between-unit heterogeneity $s_{bb, rel}$ as $u_{bb, rel}$.

4.2 Within-unit homogeneity and minimum sample size

The heterogeneity of aqueous solutions is typically considered negligible. Additionally, data accumulated from more than five earlier released CRMs using plasmid DNA in buffer solutions suggest that the likelihood of significant heterogeneity is minimal.

During the production of this specific batch, potential risks of heterogeneity were identified and proactively addressed through a series of rigorous measures, listed below.

Control of sterility conditions:

- Environment: Processing took place in a biosafety level 2 cabinet, ensuring sterile conditions for handling the solutions.
- Sterilisation: All instruments, utensils, and glassware in contact with the solution were carefully autoclaved to eliminate any contamination risk. Additionally, a complete set of sterilised equipment was used for the processing of each solution to maintain strict sterility throughout the process.

Temperature Control:

- The solution's temperature was consistently maintained between 4 °C and 6 °C throughout the filling process, using a cooling jacket around the beaker. This minimised the risk of evaporation, which could affect between-unit homogeneity.

Solution agitation:

- Continuous agitation at 1000 rpm was applied throughout the entire filling process, ensuring uniform distribution of the components.

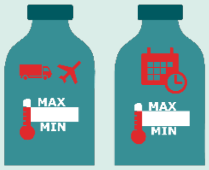
It is also worth noting that the processing time was significantly reduced compared to previous cycles, which were also not associated with heterogeneity issues.

For these reasons, conducting homogeneity tests on this batch was deemed unnecessary, as the implemented measures ensured the solution's quality and consistency.

The smallest sample intake that still yielded results with acceptable precision in the studies was taken as minimum sample size. The minimum sample size was established at 3.9 µL based on cdPCR platform and the technically valid results of the homogeneity and stability studies of a previous, sufficiently similar ruminant plasmid DNA CRM ERM-AD482 [19].

5 Stability

Box 4. Stability assessment



Stability testing is necessary to establish the conditions for storage as well as the transport conditions of the RMs to the customers. During transport, especially in summer, temperatures up to 60 °C can be reached, and stability under these conditions must be demonstrated if the RMs are to be transported without any additional cooling.

Time, temperature and light (including ultraviolet radiation) were regarded as the most relevant parameters on the stability of the materials.

The influence of ultraviolet and visible light is minimised by storing the materials in packaging, preventing light exposure. In addition, materials are stored in the dark and transported in boxes, thus removing any possibility of degradation by light.

The similarities between both materials ERM-AD482 and ERM-AD482k allow us to apply the conclusion drawn from the ERM-AD482 study [19], especially those related to testing at 4 °C, which were then not addressed in this study. Therefore, only the influence of time was investigated.

The transport stability study and the storage stability study were both carried out at a single testing temperature (-20 °C) using an isochronous design [20]. In this approach, units were stored for a particular length of time at -20 °C. Afterwards, the units were moved to conditions where further degradation can be assumed negligible (reference conditions: -70 °C). At the end of the isochronous storage, the samples were analysed simultaneously under repeatability conditions. Analysis of the material (after various exposure times) under repeatability conditions greatly improves the sensitivity of the stability tests.

5.1 Transport stability

The optimised conditions for the transport of the material to the customers (transportation on dry ice) were established during the production of the material ERM-AD482 as described by Corbisier *et al.* [19]. This study was conducted on samples which were stored at +4 °C and -20 °C for 0, 1, 2 and 4 weeks (for each testing temperature). The reference temperature was set to -70 °C. Four units per storage time were selected using a random stratified sampling scheme. Each unit was measured once by cdPCR. The measurements were performed under repeatability conditions and in a randomised sequence to be able to separate a potential analytical drift from a trend over storage time. As a conclusion, none of the trends was statistically significant on a 95 % CI at -20 °C and the material shall be shipped frozen.

Building on the results of the previous study on the ERM-AD482 material, a simplified short-term stability assessment was performed on the ERM-AD482k material. The study involved testing the material at a single temperature of -20 °C over various time intervals. Samples were stored at -20 °C for 0, 1, 2, and 4 weeks to assess stability over time. The reference temperature was set to -70 °C. Four units per storage time were selected using a random stratified sampling scheme. From each unit, five samples were measured by ddPCR. The measurements were performed under repeatability conditions and a randomised sequence was used to differentiate any potential drift in the measurement results from a potential trend over storage time.

The results of this study were screened for outliers using the single and double Grubbs test on a confidence level of 99 %.

In addition, the data were evaluated against time, and regression lines of copy number concentration versus time were calculated, to test for potential increases or decreases copy number concentration due to shipping conditions. The slopes of the regression lines were tested for statistical significance.

The results of the measurements are shown in Annex 3. The results of the statistical evaluation of the short-term stability study are summarised in Table 3.

Table 3. Results of the short-term stability study.

CRM-code	Number of individual outlying results 1)	Significance of the trend 2)
	-20 °C	-20 °C
ERM-AD482ka	none	no
ERM-AD482kb	none	no
ERM-AD482kc	none	no

1) 99 % confidence level.
2) 95 % confidence level.

Source: JRC

No technically unexplained outliers were detected for any of the ERM-AD482k materials consequently, all data were retained for the estimation of u_{sts}. None of the trends was statistically significant on a 95 % confidence level at a temperatures of -20 °C.

The material shall be shipped frozen on dry ice.

5.2 Storage stability

Storage conditions and shelf life guaranteeing the stability of the materials and the certified values were established in a long-term stability study.

To this end, for each material, four units were stored at -20 °C for 0, 4, 6 and 8 months. The reference temperature was set to -70 °C. Four units per storage time were selected using a random stratified sampling scheme. From each unit, four samples were measured by ddPCR. The measurements were performed under repeatability conditions and in a random sequence to be able to separate any potential drift in the measurement results from a potential trend over storage time.

The long-term stability data were evaluated individually. The results were screened for outliers using the single and double Grubbs test at a confidence level of 99 %.

In addition, the data were plotted against time and linear regression lines of copy number concentration versus time were calculated. The slope of the regression line was tested to assess any potential loss or increase due to storage, using regression analysis with a 95% confidence to determine statistical difference from zero. The slopes of the regression lines were significantly different from zero (95 % confidence level).

The results of the long-term stability measurements are shown in Annex 4. The results of the statistical evaluation of the long-term stability study are summarised in Table 4.

Table 4. Results of the long-term stability study.

CRM-code	Number of individual outlying results 1)	Significance of the trend 2)
	-20 °C	-20 °C
ERM-AD482ka	none	no
ERM-AD482kb	none	no
ERM-AD482kc	none	no

1) 99 % confidence level.
2) 95 % confidence level.

Source: JRC

No technically unexplained outliers were detected for any of the ERM-AD482k materials consequently, all data were retained for the estimation of u_{lts}. None of the trends was statistically significant on a 95 % confidence level for any of the temperatures. The material can therefore be stored at -20 °C.

5.3 Estimation of uncertainties

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

The uncertainties of stability during transport and storage were estimated, as described in [21]. In this approach, the uncertainty of the linear regression line with a slope of zero was calculated. The uncertainty contributions u_{sts} and u_{lts} were calculated as the product of the chosen transport time/shelf life and the uncertainty of the regression lines as:

$$u_{\text{sts, rel}} = \frac{s_{\text{rel}}}{\sqrt{\sum (t_i - \bar{t})^2}} \cdot t_{\text{tt}} \qquad \textbf{Equation 5}$$

$$u_{\text{lts, rel}} = \frac{s_{\text{rel}}}{\sqrt{\sum (t_i - \bar{t})^2}} \cdot t_{\text{sl}} \qquad \textbf{Equation 6}$$

s_{rel} RSD of all results of the stability study
t_i time elapsed at time point *i*
$\bar{t}$ mean of all t_i
t_{tt} chosen transport time (1 week at -20 °C)
t_{sl} chosen shelf life (12 months at -20 °C)

The following uncertainties were estimated:

- $u_{sts,rel}$, the uncertainty of stability during transport. This was estimated from the -20 °C studies. The uncertainty describes the possible change during a transport at -20 °C lasting for one week.
- $u_{lts,rel}$, the uncertainty of stability during storage. This uncertainty contribution was estimated from the -20 °C studies. The uncertainty contribution describes the possible degradation during storage for 12 months at -20 °C.

The results of these evaluations are summarised in Table 5.

Table 5. Uncertainties of stability during transport and storage. $u_{sts,rel}$ was calculated for a temperature of -20 °C and 1 week; $u_{lts,rel}$ was calculated for a storage temperature of -20 °C and 12 months.

CRM-code	$u_{sts,rel}$ [%]	$u_{lts,rel}$ [%]
ERM-AD482ka	0.49	2.26
ERM-AD482kb	0.60	3.93
ERM-AD482kc	1.26	8.41

Source: JRC

No significant degradation at -20 °C was observed. Therefore, the material can be shipped on dry ice.

The materials are included in the JRC's regular stability monitoring programme, to control its further stability.

Box 5. Stability monitoring

RMs are produced as batches that should last for ten years or longer. This long lifetime means that a storage stability study of limited duration cannot provide a definite "use by" date for the material. It therefore needs to be complemented by stability monitoring throughout the lifetime of the RM.

Therefore, the stability of RMs whose assigned values might change is regularly monitored. The monitoring frequency depends on the outcome of the storage stability assessment.

If the tests confirm the stability of the assigned values, the material remains on sale. If not, possible actions include the retraction of the value in question, retraction of the complete material or a change of the certified value. Customers are notified if the change is larger than the uncertainty of the assigned value.

5.4 Stability under repeated use

The impact of repeated freeze-thaw cycles on the stability of the material ERM-AD482k was investigated in the context of material degradation during these cycles.

Freeze-thaw studies were conducted on additional units produced during the processing of ERM-AD482ka, ERM-AD482kb, and ERM-AD482kc materials. All units were initially stored at -70 °C for one week. Following this period, the units were transferred to -20 °C, with the exception of two units per concentrations, which were kept at -70 °C (reference temperature). Subsequently, the selected units underwent 1, 2, 3, or 5 freeze-thaw cycles, with thawing phases conducted either at 4 °C or at 20 °C. A single unit was assigned to each condition of the study. From each unit, a sample was aliquoted, mixed with the master mix, primers and probe, and analysed in quintuplicate using ddPCR.

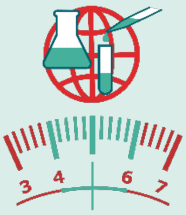
The results indicated a statistical significant decrease for several freeze-thaw cycles during which samples were thawed at 4 °C and 20 °C (data not shown).

Opened vials should be closed with the original stopper and cap after use. Under the condition that any microbial contamination and evaporation have been excluded, the solution of ERM-AD482k can be stored at (4 ± 3) °C for up to 48 hours, given that during the characterisation study, it was verified that changes observed to the certified concentrations are not significant.

Users are advised, as stated in the certificate, to avoid freeze-thaw cycles to ensure stability of the certified value. Thawing at 4 °C is recommended.

6 Characterisation

Box 6. Reference material characterisation



Material characterisation is the process of determining the property value(s) of an RM. While ISO 17034 [1] allows to characterise an RM in various ways. Quality management procedures of the JRC are more stringent and allow characterisation only by either interlaboratory comparison or the use of a primary method confirmed by independent analysis.

The material characterisation was based on an interlaboratory comparison, and the copy number concentrations of ERM-AD482ka, ERM-AD482kb and ERM-AD482kc were determined by combining independent datasets obtained in laboratories. Digital PCR can provide datasets suitable for determining the absolute value. Although the methodology is similar across the three types of digital PCR, each digital PCR differs in its application. Using an interlaboratory comparison transforms the potential bias inherent in each dataset into a random variable. By averaging over multiple datasets, the combined effect of these biases is minimised, leading to a more accurate result.

6.1 Selection of participants

Five laboratories were selected based on criteria that comprised both technical competence and quality management aspects. Each participating laboratory was required to operate a quality system and to deliver documented evidence of its laboratory proficiency in the field of digital PCR measurements in relevant matrices by submitting results from earlier interlaboratory comparison exercises or method validation reports. Having a formal accreditation was not mandatory, but meeting the requirements of ISO/IEC 17025 [3] was obligatory.

6.2 Study setup

Laboratories received two units of each concentration level of ERM-AD482k. Depending of the method applied, they were requested to measure:

- cdPCR method: 5 per unit for each dataset (10 independent results)
- ddPCR method: 10 per unit for each dataset (20 independent results)
- cddPCR method: 5 per unit for each dataset (10 independent results)

The units for material characterisation were selected using a random stratified sampling scheme and covered the whole batch.

The measurements were spread over different numbers of days depending on the applied method:

- cdPCR method: 3 days
- ddPCR method: 2 days
- cddPCR method: 3 days

For each dataset, a no template control (NTC) was included to verify the possible contamination occurring during the analysis. The results of this sample were used to evaluate the quality of the analysis.

Additionally, a unit of ERM-AD482 as a blind quality control (QC) sample was provided. The results for this sample were used to support the evaluation of the characterisation results.

6.3 Methods and Measurement procedures used

The characterisation is based on an interlaboratory comparison using three different digital PCR techniques as detailed in Annex 5. Master mix, primers and probe concentrations are specified in Table 5.1, (Annex 5) and thermocycling conditions in Table 5.2, (Annex 5). The PCR reaction, designed to amplify the bovine target, was then carried out using each of the digital PCR platforms.

The laboratory code (e.g., D01) is assigned randomly and does not reflect the order of the laboratories listed in Section 2.6. Each lab method code comprises a numerical identifier assigned to the laboratory followed by an abbreviation of the specific measurement technique employed (e.g., D01-ddPCR).

All participating laboratories used digital PCR to measure copy number concentrations. While some laboratories implemented a unique digital PCR method, others employed a combination of the three digital PCR methods.

It finally resulted in a balanced distribution among the three types of techniques, with each technique represented with three data sets.

6.4 Evaluation of results

The characterisation study resulted in nine datasets per concentration. All individual datasets of the participating laboratories, grouped per concentration, are displayed in Table 6.1, 6.2 and 6.3 in Annex 6.

6.4.1 Technical evaluation

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

- compliance with the instructions given:

  Arrival of the sample under freezing conditions

  Storage of the samples at -20 °C upon arrival and at 4 °C after thawing

  Measurements performed on the number of days prescribed and the measurement sequence
- Blank (NTC) is below 1 cp/µL
- method performance: agreement of the measurement results with the assigned value of the QC sample

Based on the above criteria, no dataset had to be rejected.

6.4.2 Statistical evaluation

The accepted datasets were tested for normality of dataset means using skewness tests and normal probability plots and were tested for outlying means using the Grubbs test and using the Cochran test for outlying standard deviations (both at a 99 % confidence level). Standard deviations within (s_{within}) and between ($s_{between}$) laboratories were calculated using one-way ANOVA. The results of these evaluations are shown in Table 6 and 7.

Table 6. Statistical evaluation of the technically accepted datasets for ERM-AD482k.

CRM-code	p [1)]	Outliers		Normally distributed	Statistical parameters			
		Means	Variances		Mean [cp/µL]	s [cp/µL]	$s_{between}$ [cp/µL]	s_{within} [cp/µL]
ERM-AD482ka	9	no	yes	yes	141.35	9.77	9.61	9.25
ERM-AD482kb	9	no	yes	yes	38.09	3.11	2.89	4.10
ERM-AD482kc	9	no	yes	yes	9.55	0.97	0.75	2.32

1) number of technically valid datasets.

Source: JRC

The dataset means follow normal distributions. None of the data contains outlying means, but variances inhomogeneity between the datasets was identified for all materials. Using one-way ANOVA, significant differences among the data at both 95 % and 99 % confidence levels were observed for all materials, with the exception of the ERM-AD482kc, which did not show significant differences at the 99 % confidence level. This simply underscores the reality that various methods possess distinct intrinsic characteristics, depending on the specific approach employed. Nevertheless, the data has been organised according to each method and subjected to statistical analysis to identify any potential method-dependent behaviours. (Table 7, 8 and 9).

Table 7. Statistical evaluation of the technically accepted datasets for ERM-AD482ka depending of the method.

ERM-AD482ka	p [1)]	Outliers		Normally distributed	Statistical parameters	
		Means	Variances		Mean [cp/µL]	s [cp/µL]
ddPCR	6	no	no	N/A*	130.63	4.00
cdPCR	6				149.61	8.50
cddPCR	6				143.84	5.89

1) number of technically valid datasets.

*: not enough dataset to determine the normality.

Source: JRC

Table 8. Statistical evaluation of the technically accepted datasets for ERM-AD482kb depending of the method.

ERM-AD482kb	p [1)]	Outliers		Normally distributed	Statistical parameters	
		Means	Variances		Mean [cp/µL]	s [cp/µL]
ddPCR	6	no	no	N/A*	34.98	0.94
cdPCR	6				40.53	3.68
cddPCR	6				38.70	2.57

1) number of technically valid datasets.

*: not enough dataset to determine the normality.

Source: JRC

Table 9. Statistical evaluation of the technically accepted datasets for ERM-AD482kc depending of the method.

ERM-AD482kb	p [1)]	Outliers		Normally distributed	Statistical parameters	
		Means	Variances		Mean [cp/µL]	s [cp/µL]
ddPCR	6	no	no	N/A*	8.52	0.44
cdPCR	6				10.13	0.86
cddPCR	6				10.04	1.38

1) number of technically valid datasets.

*: not enough dataset to determine the normality.

Source: JRC

The *t*-tests indicate that the datasets are incompatible in pairs, revealing significant differences between the data generated by ddPCR and those obtained from cdPCR and cddPCR across all materials. This discrepancy underscores the inherent variability of different methodologies, which stems from the unique characteristics of each platform employed.

Nevertheless, all measurement methods were considered technically sound and all results were retained, as these standards are used to establish a cut-off value by qPCR methods, which are significantly less sensitive than digital PCR techniques. Consequently, a single certified value is assigned for each material ERM-AD482ka, ERM-AD482kb and ERM-AD482kc.

The uncertainty related to the characterisation is estimated as the standard error of the mean of means ($s/\sqrt{p}$) by combining the standard uncertainties of each dataset mean (Table 10).

Table 10. Uncertainty of characterisation for ERM-AD482k.

CRM-code	p [1)]	Mean [cp/µL]	s [cp/µL]	u_{char} [cp/µL]	u_{char} [%]
ERM-AD482ka	9	141.35	9.78	3.26	2.30
ERM-AD482kb	9	38.09	3.11	1.04	2.73
ERM-AD482kc	9	9.55	0.97	0.32	3.39

1) number of technically valid datasets.

Source: JRC

7 Value Assignment

Box 7. Assignment of values to a reference material

Based on the outcome of characterisation measurements three types of values can be assigned, namely certified, indicative or additional material information values.

Certified values are values that fulfil the highest standards of accuracy. Procedures at JRC Directorate F require a sufficient number of datasets to assign certified values. Full uncertainty budgets in accordance with ISO 17034 [1] and ISO 33405 [2] are required. Certified values of a CRM can be used for calibration and trueness controls as well as for quality control measures.

Indicative values are values where either the uncertainty is deemed too large or too few independent datasets are available to allow certification. Indicative values of an RM can be used for statistical quality control (homogeneity and stability have been assessed) but not for calibration, demonstration of method or laboratory proficiency or method trueness.

Additional material information values are values for which homogeneity and stability have usually not been assessed and only insufficient data for characterisation is available. Consequently, an estimate of the reliability of the values is not possible and no uncertainty is given. Additional material information values cannot be used for calibration, demonstration of method or laboratory proficiency or method trueness. They can be used to e.g. anticipate possible interferences in measurement processes.

7.1 Certified values and their uncertainties

The unweighted mean of the means of the accepted datasets as shown in Table 11 was assigned as certified value for each parameter.

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

$$U_{CRM,\ rel} = k \cdot \sqrt{u_{bb,\ rel}^2 + u_{sts,\ rel}^2 + u_{lts,\ rel}^2 + u_{char,\ rel}^2} \qquad \textbf{Equation 7}$$

- u_{char} was estimated as described in Section 6.4
- u_{bb} was estimated as described in Section 4.1
- u_{sts}, and u_{lts} were estimated as described in Section 5.3

Following JRC's procedures for assigning uncertainties to certified values, a coverage (k) factor of 2 can be chosen if the main uncertainty component has at least five degrees of freedom. As can be seen in Table 11, the $u_{lts,rel}$, the $s_{bb,\ rel}$ and $u_{char,\ rel}$ are the dominant contributions to the combined uncertainty and it has eight degrees of freedom. Therefore, a k-factor of 2 was applied to obtain the expanded uncertainties.

The certified values and their uncertainties are summarised in Table 11.

Table 11. Certified values and their uncertainties for ERM-AD482k.

CRM-code	Certified value [cp/μL]	$u_{char, rel}$ [%]	$s_{bb, rel}$ [%]	$u_{sts, rel}$ [%]	$u_{lts, rel}$ [%]	$U_{CRM, rel}$ [%]	U_{CRM} [1)] [cp/μL]
ERM-AD482ka	141.35	2.30	1.44	0.49	2.26	7.84	11.07
ERM-AD482kb	38.09	2.73	2.53	0.60	3.93	12.92	4.92
ERM-AD482kc	9.55	3.39	5.00	1.26	8.41	23.92	2.28

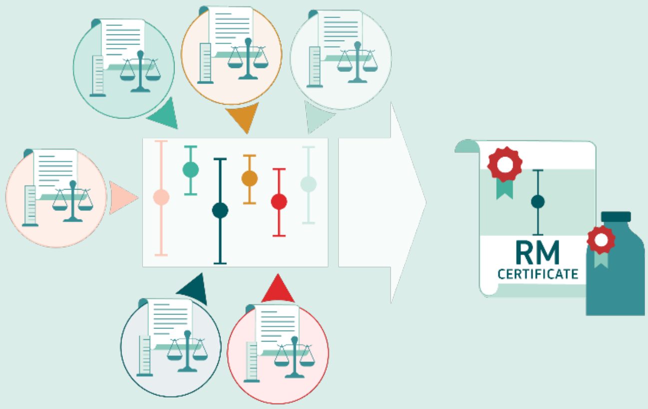
1) Expanded (k = 2) and rounded uncertainty; uncertainties are always rounded up [22] and in a way that the rounding error corresponds to 3 % to 30 % of the uncertainty.

Source: JRC

8 Metrological traceability and commutability

8.1 Metrological traceability

Box 8. Metrological traceability



Metrological traceability of measurement results is a key requirement for ensuring the comparability of data. As CRMs are used to make measurement results traceable to a common reference, metrological traceability of its certified values to a stated reference is essential.

The certified value of a CRM is metrologically traceable if the measurement results used for establishing it can all be related to the same reference through an unbroken chain of calibrations.

This requires that these measurements

- refer to the same property (e.g. Pb content) and the same (kind of) quantity (e.g. Pb mass fraction),
- result in a number and its uncertainty (e.g. 6 ± 2) expressed in the same measurement unit (e.g. µg/kg).

The concept of metrological traceability rests in chemistry on several anchor points, namely identity, quantity value and measurement unit. The identity of a measurand can be defined by its chemical structure alone or can be operationally defined, the quantity value of the measurand can refer to the SI or to other appropriate references.

8.1.1 Identity

The ruminant DNA plasmid is a structurally defined analyte and its identity was confirmed by sequencing. The data collected during the characterisation were obtained by applying digital PCR methods for the determination of the final concentrations. The plasmid identity is structurally defined; however, since the certified values were established solely using digital PCR methods, the measurand is considered operationally defined (Annex 5 table 5.1).

8.1.2 Quantity value

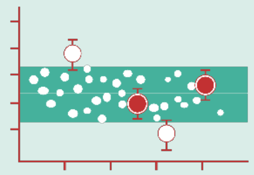
Traceability of the obtained results is based on the traceability of all relevant input factors. Investigation of the method and measurement details of the individual results show that all relevant input parameters of each technically accepted dataset have been properly calibrated. All technically accepted datasets are therefore traceable to the same reference, namely the SI. This traceability to the same reference is also confirmed by the agreement of results within their respective uncertainties. As the assigned values are combinations of agreeing results individually traceable to the SI, the assigned quantity values themselves are traceable to the SI as well.

8.2 Commutability

Box 9. Commutability

Commutability is a prerequisite for RMs intended to be used for calibration or quality control of different measurement procedures targeting the same measurand. The concept of commutability of a RM is defined by the VIM [12] as:

"*property of a reference material, demonstrated by the closeness of agreement between the relation among the measurement results for a stated quantity in this material, obtained according to two given measurement procedures, and the relation obtained among the measurement results for other specified materials*"



Commutability is a property of an RM indicating how well an RM mimics the characteristics of a typical routine sample in various measurement procedures for a stated measurand.

The same RM may be commutable for some measurement procedures but non-commutable for others. A commutability statement is therefore only valid for the mentioned measurement procedure(s).

The structure of the ruminant plasmid ERM-AD482 is defined via its DNA sequence.

The certified values of the ERM-AD482k are operationally defined and can only be obtained when tested in accordance with the digital PCR methods described in Annex 5.

The CRMs are intended to determine the cut-off value by qPCR measurements of ruminant DNA in feed. Consequently, in combination with the measurement method validated by the EURL-AP [10], this set of CRMs is establishing the arbitrary reference system required for the detection of ruminant PAPs.

Commutability of this reference material does not need to be assessed.

9 Instructions for use

9.1 Safety information

The usual laboratory safety measures apply.

9.2 Storage conditions

Unopened units should be stored at (-20 ± 5) °C in the dark.

The user should close any unit immediately after taking a sample.

Once defrosted, the materials can be stored for 48 hours at (4 ± 3) °C.

Users are advised to avoid freeze-thaw cycles.

If a user intends to use the materials after additional freeze–thaw cycles, it is the user's responsibility to verify that the materials are stable under these conditions.

For more information regarding the shelf life of reference materials please consult ERM Application Note 7 [23].

Note that the European Commission cannot be held responsible for changes that may happen to samples after opening or when the material is stored differently from the stated storage conditions at the customer's premises.

9.3 Use of the material

After the completeness of thawing step, the material must be gently re-homogenised by repeatedly turning over the units and shaking for 1 min at 1000 rpm at 20 °C. The materials are ready to use.

For general information on handling of reference materials, please consult ERM Application Note 6 [24].

9.4 Minimum sample size

The minimum sample size is 3.9 µL.

9.5 Use of the certified values

Intended use

These materials are used to determine a cut-off value of qPCR as defined in the standard operating procedure, developed by the European Union Reference Laboratory for Animal Proteins in feeding stuffs (EURL-AP-SOP-Ruminant-PCR-V1.3.pdf (craw.eu)). The set should be used to construct calibration curves at low copy number concentrations to determine a cut-off value. This cut-off value shall only be used in quantitative real-time PCR to discriminate feed samples negative for ruminant animal proteins from samples positive for ruminant animal proteins.

Comparing a measurement result with the certified value

A result is unbiased if the combined standard uncertainty of measurement and certified value covers the difference between the certified value and the measurement result (see also ERM Application Note 1 [25].

When assessing the method performance, the measured values of the CRMs are compared with the certified values. The procedure is summarised here:

- Calculate the absolute difference between mean measured value and the certified value (Δ_{meas}).
- Combine the measurement uncertainty (u_{meas}) with the uncertainty of the certified value (u_{CRM}): $u_\Delta = \sqrt{u_{meas}^2 + u_{CRM}^2}$
- Calculate the expanded uncertainty (U_Δ) from the combined uncertainty (u_Δ) using an appropriate coverage factor, corresponding to a level of confidence of approximately 95 %.
- If $\Delta_{meas} \leq U_\Delta$ then no significant difference exists between the measurement result and the certified value, at a confidence level of approximately 95 %.

Use in quality control charts

The material(s) can be used for quality control charts. Using CRMs for quality control charts has the added value that a trueness assessment is built into the chart.

10 Conclusions

ERM-AD482ka, ERM-AD482kb and ERM-AD482kc are reference materials certified for their copy number concentrations of plasmid DNA bearing a ruminant-specific DNA fragment. These materials were produced and certified in accordance with ISO 17034:2016 [1] and ISO 33405:2024 [2], and under the scope of ISO 17034 accreditation.

The intended use of these materials is to determine a cut-off value of quantitative PCR as defined in the standard operating procedure, developed by the European Union Reference Laboratory for Animal Proteins in feedingstuffs (EURL-AP-SOP-Ruminant-PCR-V1.3.pdf (craw.eu) [11]). The set should be used to construct calibration curves at low copy number concentrations to determine a cut-off value. This cut-off value shall only be used in quantitative real time PCR to discriminate feed samples negative for ruminant animal proteins from samples positive for ruminant animal proteins.

The following values were assigned:

Table 12. Values assigned to ruminant plasmid DNA in buffered solution, ERM-AD482ka, ERM-AD482kc and ERM- AD482kc.

Certified values		
	Copy number concentration 2) [cp/µL]	Uncertainty 3) [cp/µL]
ERM-AD482ka 1)	141	11
ERM-AD482kb 1)	38	5
ERM-AD482kc 1)	9.6	2.0

1) As obtained by chamber digital PCR (cdPCR), droplet digital PCR (ddPCR) and crystal droplet digital PCR (cddPCR) methods using the ruminant-specific primers and probe described in the standard operating procedure developed by the European Union Reference Laboratory for Animal Proteins in feedingstuffs [https://www.eurl.craw.eu/wp-content/uploads/2021/05/EURL-AP-SOP-Ruminant-PCR-V1.3.pdf]

2) Certified values are values that fulfil the highest standards of accuracy. The given values represent the unweighted mean value of the means of accepted sets of data, each set being obtained in a different laboratory and/or with a different method of determination. The certified value and its uncertainty are traceable to the International System of Units (SI).

3) The uncertainty of the certified value is the expanded uncertainty with a coverage factor k = 2 corresponding to a level of confidence of 95 %, estimated in accordance with ISO 17034:2016 and ISO 33405:2024.

Source: JRC

List of abbreviations

ANOVA	Analysis of variance
bp	Base pair
c_{DNA}	DNA mass concentration
cdPCR	Chamber digital PCR
cddPCR	Crystal droplet digital PCR
cp	Copies
CRA-W	Walloon Agricultural Research Centre
CRM	Certified reference material
Cq	Quantification cycle (also referred to as threshold cycle, Ct)
DNA	Deoxyribonucleic acid
ddPCR	Droplet digital PCR
dPCR	Digital PCR
EC	European Commission
EDTA	disodium dihydrogen ethylenediaminetetraacetate
ERM®	Trademark owned by the European Commission; used by the JRC for reference materials
EU	European Union
EURL-AP	European Union Reference Laboratory for the detection of Animal Proteins in feedingstuffs
GUM	Guide to the Expression of Uncertainty in Measurement
ISO	International Organization for Standardization
JRC	Joint Research Centre of the European Commission
k	Coverage factor
LB	Luria Bertani
LOD	Limit of detection
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 replicate analysis per unit
N	Number of units analysed
n.a.	Not applicable
N_A	Avogadro constant [cp/mol]
n.c.	Not calculated
NTC	No template control
p	Number of technically valid datasets
PAP	Processed animal protein
PCR	Polymerase chain reaction

QC	Quality control
qPCR	Quantitative PCR
rel	Index denoting relative figures (uncertainties etc.)
RM	Reference material
RSD	Relative standard deviation
r^2	Coefficient of determination of the linear regression
s	Standard deviation
s_b	Standard deviation of the slope of the regression line
s_{bb}	Between-unit standard deviation; an additional index "rel" is added when appropriate; this parameter is linked to the homogeneity of the material
s_{between}	Standard deviation between groups as obtained from ANOVA; an additional index "rel" is added as appropriate
SI	International System of Units
s_{wb}	Within-unit standard deviation; this parameter is linked to the homogeneity of the material
s_{within}	Standard deviation within groups as obtained from ANOVA; an additional index "rel" is added as appropriate
t	Time
$\bar{t}$	Mean of all time
t_i	Time point for each replicate
t_{sl}	Chosen shelf life
t_{tt}	Chosen transport time
TE	Buffer containing TRIS, 0.01 mmol/L EDTA at pH 8.0
TRIS	2-amino-2-(hydroxymethyl)propane-1,3-diol hydrochloride
u	Standard uncertainty
U	Expanded 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_{char}	Standard uncertainty of the material characterisation; an additional index "rel" is added as appropriate
u_{CRM}	Combined standard uncertainty of the certified value; an additional index "rel" is added as appropriate
U_{CRM}	Expanded uncertainty of the certified value; an additional index "rel" is added as appropriate
u_{d}	Standard uncertainty of the correction of the measurement bias between long-term stability studies.
u_{lts}	Standard uncertainty of the long-term stability; an additional index "rel" is added as appropriate
u_{meas}	Standard measurement uncertainty

u_{sts}	Standard uncertainty of the short-term stability; an additional index "rel" is added as appropriate
UNG	Uracil-N-glycosylase
$\overline{x}$	Arithmetic mean
Δ_{meas}	Absolute difference between mean measured value and the certified value
ν_{MSwithin}	Degrees of freedom of MS_{within}
$\overline{y}$	Mean of all results of the study

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Annexes

Annex 1. Identity confirmation: sequence alignment results

“Query” represents the sequence obtained by Sanger sequencing for identity confirmation.

“Sbjct” represents the reference sequence as obtained from the EURL-AP.

The insert in pUC18 plasmid is represented in bold and bigger format.

The 86 bp ruminant DNA fragment is highlighted in yellow, green and black.

The primers are underlined and highlighted in green.

The probe is represented highlighted in black with white font.

Outcome of the alignment information:

- A gap is located at the 431 position (indicated in red). This gap located into the insert does not affect the fragment of interest for PCR assays.
- A mismatch is located at 2237 position (indicated in blue)

➢

⇨ Identities: 2859/2860 (0.03 %) and Gap: 1/2860 (0.03 %) => 99.94 % overall identity.

```
Query  1    GCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCA  60
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Sbjct  1    GCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCA  60

Query  61   CGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCT  120
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Sbjct  61   CGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCT  120

Query  121  CACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAAT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  181  TGTGAGCGGATAACAATTTCACACAGGAAACAGCTATGACCATGATTACGAATTCGAGCT  240
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Query  601  GCAGCCTGAATGGCGAATGGCGCCTGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTA  660
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Sbjct  720  AGCCCCGACACCCGCCAACACCCGCTGACGCGCCCTGACGGGCTTGTCTGCTCCCGGCAT  779
```

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Sbjct  2820  CGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGA  2859
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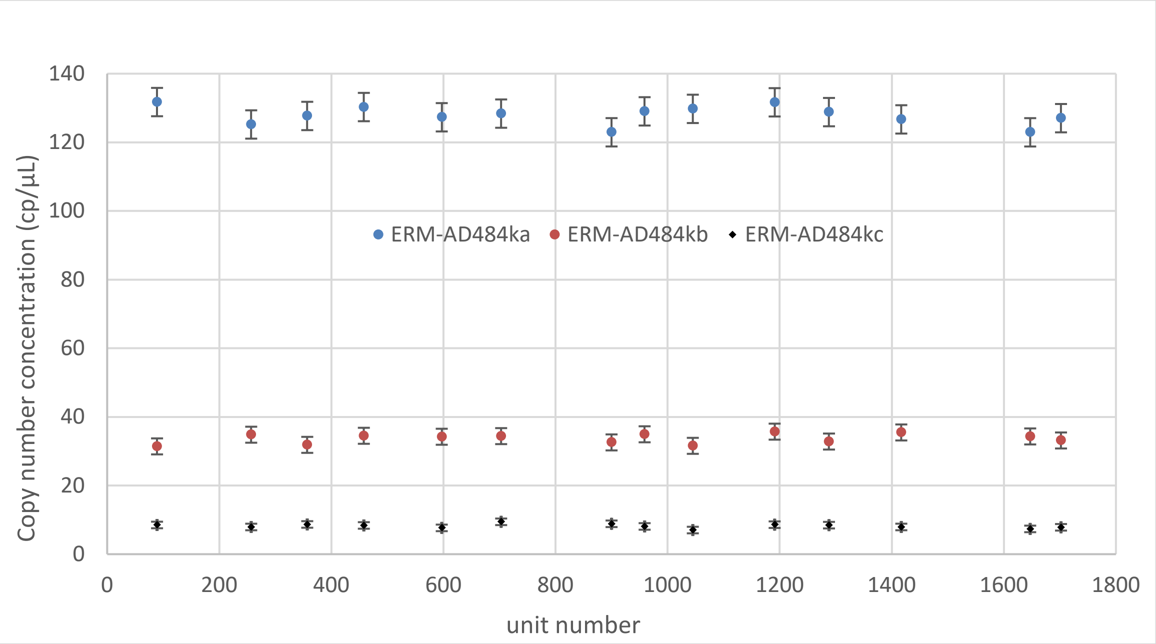
Annex 2. Results of the homogeneity measurements

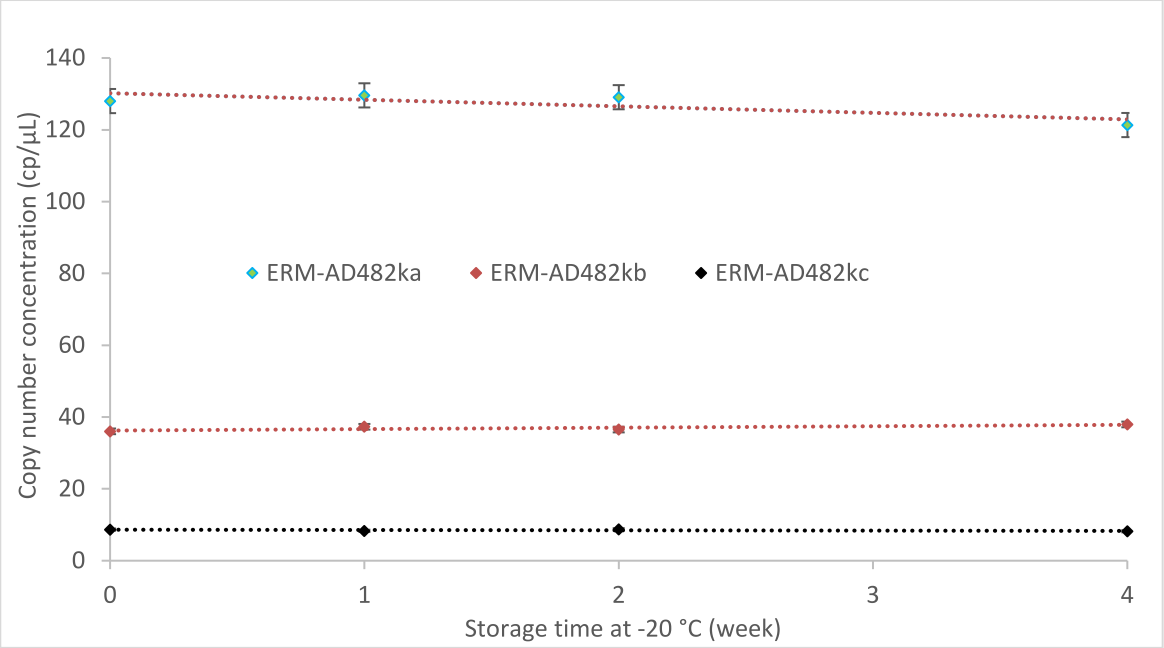
Figure 2.1. ERM-AD482ka in blue, ERM-AD482kb in orange and ERM-AD482kc in black. Each dot represents the mean of 1 unit (measured 6 times) collected every 128 units along the processing. The error bars represent the 95 % confidence interval based on the within-group standard deviation (s_{wb}) from one-way ANOVA for all units (measurements/replicates) of the homogeneity study.



Source: JRC

Annex 3. Results of the transport stability measurements

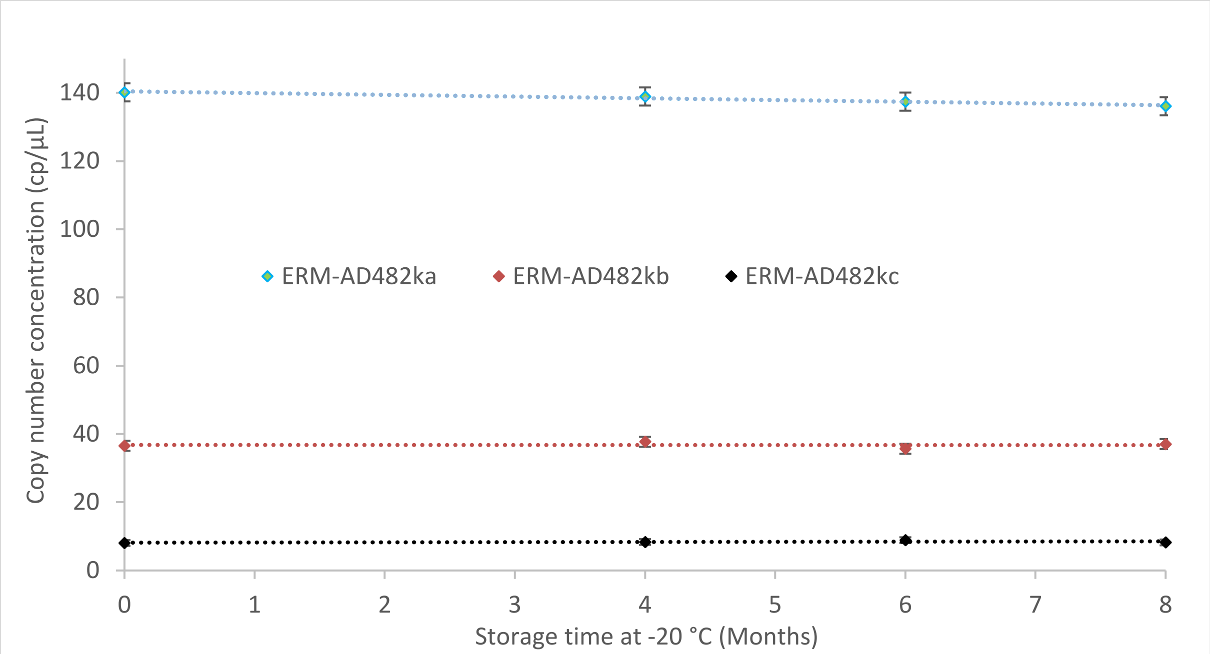
Figure 3.1. ERM-AD482ka in blue, ERM-AD482kb in orange and ERM-AD482kc in black. Each dot represents the mean of the means of the four units (measured 4 times) per storage time. The error bars represent the 95 % confidence interval of the mean of the four units based on the within-group standard deviation (s_{wb}) as obtained by one-way ANOVA.



Source: JRC

Annex 4. Results of the storage stability measurements

Figure 4.1. ERM-AD482ka in blue, ERM-AD484kb in orange and ERM-AD484kc in black. Each dot represents the mean of the four units (measured 4 times) per storage time. The error bars represent the 95 % confidence interval based on the within-group standard deviation (s_{wb}) from one-way ANOVA for all measurements of the storage stability study.



Source: JRC

Annex 5: Summary of methods used in the characterisation study

During this characterisation study, three platforms were used, each of them generating three sets of data.

- Chamber digital PCR from Fluidigm (cdPCR)

The protocol outlined was used for all cdPCR measurements conducted at JRC (Geel, BE), as well as by all laboratories participating in the characterisation study. The primers and probe were supplied by JRC (refer to Table B1 for additional details).

All volumes were gravimetrically measured. Specifically, 3.9 µL of the undiluted DNA sample (ERM-AD482ka, ERM-AD482kb, or ERM-AD482kc) was combined with 6.1 µL of a pre-sample mix solution and introduced into a well of the 12.765 Digital Array™ IFCs, which was primed with control line fluid. The 6.1 µL pre-sample mix solution contained the primers and probe targeting the bovine target at the final concentrations specified in Table 6.1, along with 2x Brilliant II QPCR Master Mix (ref: 600805) from Agilent and 20x Fast GT sample loading reagent from Fluidigm. Amplification was performed in accordance with the parameters detailed in Table 6.2, and an image of the digital array was captured at the conclusion of each cycle.

To generate the results, the software applied a quality threshold of 0.4 for automatic partition calling, employed linear baseline correction, utilized the auto (global) Ct threshold method, and established a target Ct interval between 20 and 50.

- Droplet digital PCR from Bio-Rad (ddPCR)

The protocol described was used for all ddPCR measurements at JRC (Geel, BE), as well as by all the laboratories performing ddPCR measurements in the characterisation study. The primers and probe were provided by the JRC (Geel, BE) (refer to Table B1 for more information).

All volumes were gravimetrically measured. A volume of 7.7 µL of the DNA samples (ERM-AD482ka, ERM-AD482kb or ERM-AD482kc) were mixed with 14.3 µL of pre-sample mix solution. The pre-sample mix solution contains the primers and probes for the ruminant target at final concentrations as outlined in Table 6.1, together with 2x ddPCR Supermix for Residual DNA quantification from Bio-Rad (Bio-Rad Laboratories N.V., Temse, BE). This mixture was loaded into DG8™ cartridges (Bio-Rad) for a manual droplet generation or into 96-well PCR plate (Bio-Rad) when using an Auto Droplet Generator (Auto DG). In both cases, when generated the droplets were placed into a 96-well PCR plate. The amplification was performed according to the specifications in Table 6.2. After amplification, the droplets were read and the results analysed with the QuantaSoft software using the appropriate threshold for each analysis.

In this specific case, laboratories were allowed to determine droplet volumes if they had the capability to measure them internally.

One laboratory reported that when using the "ddPCR Supermix for Residual DNA Quantification" master mix, the measured value fell within the measurement uncertainty for the "ddPCR supermix (no dUTP)". This laboratory established a droplet volume of (0.788 ± 0.028) nL, representing the mean and the expanded uncertainty at a 95 % confidence level.

Given that the droplet volume for the "ddPCR Supermix for Residual DNA Quantification" master mix is considered equivalent to that of the "ddPCR supermix (no dUTP)", another laboratory used its internally measured volume of 0.771 nL as the droplet volume.

The third laboratory opted for the default volume recommended by the supplier, which is 0.795 nL.

As a result, despite the different droplet volumes used by the three laboratories, all values were consistent with the target volume established for this study: (0.788 ± 0.028) nL.

Criterion applied for excluding results:

1. When less than 10000 droplets were generated.
2. When the separation between positive and negative droplets was equivocal (meaning that there was no clear separation between both populations).

- Crystal digital PCR from Naica (cddPCR)

The protocol outlined herein was employed consistently across all laboratories conducting cddPCR measurements during the characterization study. Primers and probes were supplied by the JRC (Geel, BE) (refer to Table 5.1 for additional details). Notably, in this instance, the probe's quencher (Tamra) has been substituted with QSI.

All volumes were gravimetrically measured. Specifically, 5.2 µL of DNA samples (ERM-AD482ka, ERM-AD482kb, or ERM-AD482kc) were combined with 20.8 µL of a pre-sample mix solution. This pre-sample mix contained primers and probes targeting ruminant's genes at the final concentrations specified in Table 5.1, along with 2x PerfeCTa® qPCR ToughMix® without Rox from Quantabio and 28.1 µL of fluorescein. The resulting mixture was then loaded into Sapphire chips, which were subsequently placed in the Geode, an automated droplet generator and thermocycler. Amplification was carried out according to the parameters set forth in Table 5.2.

Following amplification, the droplets were analysed, and the results were interpreted using Crystal Miner software, applying the appropriate thresholds for each analysis.

Table 5.1. Primers and probe used to amplify the bovine target.

Primer/ probe	Sequence	Concentration in PCR reaction [nM]	Amplicon size [bp]
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	

*: for cddPCR, the quencher, TAMRA is replaced by QSI.

Source: JRC

Table 5.2. Thermal cycling protocol used for the simplex PCR reaction amplifying the bovine target.

Step	Time [s]	Temperature [°C]	Repeats
UNG incubation	120	50	1
Polymerase activation	600	95	1
DNA denaturation	15	95	50
Annealing / extension	60	60	
Hold	infinite	4	1

Source: JRC

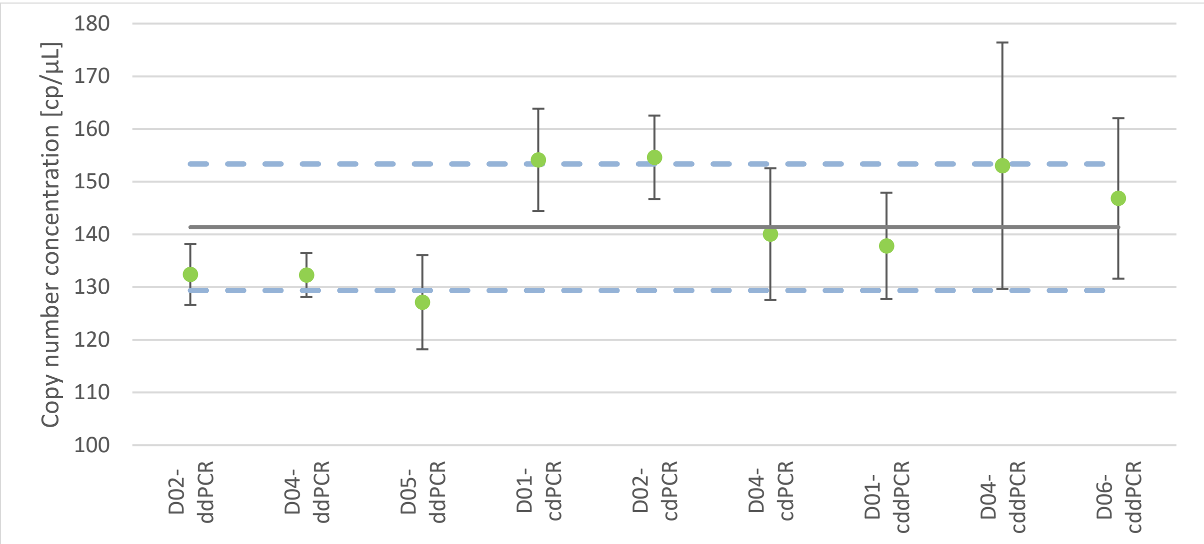
Annex 6. Results of the characterisation measurements

Table 6.1. Individual results of the characterisation measurements of ERM-AD482ka.

Dataset code / Replicates [cp/µL]	D02-ddPCR	D04-ddPCR	D05-ddPCR	D01-cdPCR	D02-cdPCR	D04-cdPCR	D01-cddPCR	D04-cddPCR	D06-cddPCR
replicate 1	124.8	132.2	137.7	164.7	150.7	147.8	139.9	147.8	153.8
replicate 2	121.9	132.5	144.4	158.2	149.9	131.0	120.4	155.5	141.6
replicate 3	129.8	133.9	136.9	159.0	157.1	133.1	144.4	212.9	150.4
replicate 4	122.8	131.1	135.1	154.9	164.0	126.3	121.4	133.1	133.3
replicate 5	133.0	129.7	137.6	153.8	160.5	137.5	149.9	167.6	140.7
replicate 6	136.9	127.2	111.5	159.3	142.7	159.2	138.5	149.5	179.4
replicate 7	131.8	129.5	115.3	141.7	159.2	149.1	138.5	144.6	137.3
replicate 8	128.6	126.0	122.4	143.4	166.6	157.7	139.3	143.4	147.9
replicate 9	139.1	139.5	134.1	139.3	147.8	132.9	149.8	143.0	158.9
replicate 10	129.4	131.9	133.3	167.3	147.8	125.8	136.2	133.0	125.0
replicate 11	139.9	132.1	120.5						
replicate 12	138.7	131.1	123.4						
replicate 13	139.3	130.8	127.0						
replicate 14	132.4	124.4	114.7						
replicate 15	142.8	139.0	133.4						
replicate 16	134.3	137.5	122.6						
replicate 17	129.0	132.3	123.3						
replicate 18	133.4	137.0	124.4						
replicate 19	130.4	136.1	123.0						
replicate 20	130.1	*	121.7						
Mean	**132**	**132**	**127**	**154**	**155**	**140**	**138**	**153**	**147**
SD [cp/µL]	**6**	**4**	**9**	**10**	**8**	**12**	**10**	**23**	**15**
*: measurement result excluded from the study due to technical reasons.									

Source: JRC

Figure 6.1. Characterisation data for the ERM-AD482ka, the continuous line represents the certified value, the set of dashed lines reflects the certified range and the error bars represent the standard deviation of the measurements.



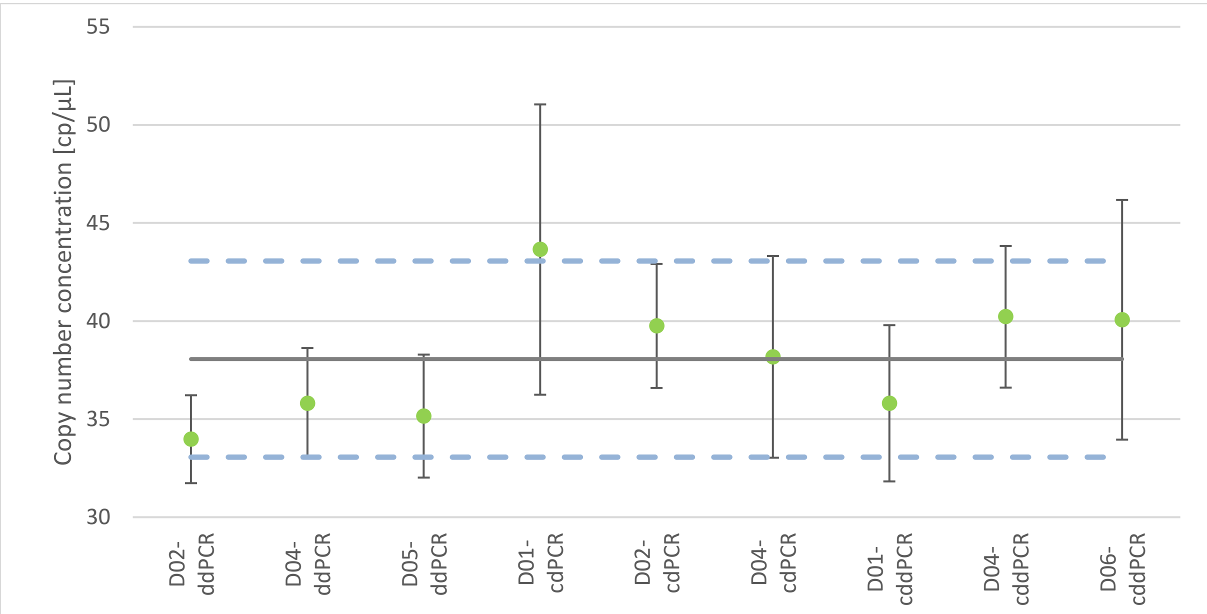
Source: JRC

Table 6.2. Individual results of the characterisation measurements of ERM-AD482kb.

dataset code / Replicates [cp/µL]	D02-ddPCR	D04-ddPCR	D05-ddPCR	D01-cdPCR	D02-cdPCR	D04-cdPCR	LD1-cddPCR	D04-cddPCR	D06-cddPCR
replicate 1	33.5	33.0	42.0	44.4	41.0	45.0	35.1	37.5	44.8
replicate 2	34.5	30.9	39.3	35.3	40.1	44.9	34.3	40.3	43.8
replicate 3	35.1	33.6	36.9	41.1	40.3	41.0	35.9	34.8	31.7
replicate 4	32.6	34.1	29.7	36.3	33.5	41.8	29.7	41.6	44.0
replicate 5	32.9	36.1	33.5	56.8	35.7	37.3	43.5	40.8	43.9
replicate 6	34.1	39.7	37.1	45.5	38.7	30.1	36.3	39.2	32.4
replicate 7	32.6	36.5	36.1	39.6	42.0	31.3	30.4	38.9	40.5
replicate 8	37.5	34.7	34.4	42.6	39.8	38.2	39.4	38.3	33.5
replicate 9	32.3	38.2	34.3	39.0	41.9	37.2	37.1	48.4	36.6
replicate	31.9	39.1	34.1	55.8	44.5	34.9	36.3	42.3	49.3
replicate	35.0	36.5	32.8						
replicate	34.1	37.1	28.4						
replicate	32.7	37.8	33.6						
replicate	34.0	37.6	33.9						
replicate	36.3	33.5	37.3						
replicate	34.3	33.5	33.0						
replicate	40.0	36.3	38.7						
replicate	29.4	37.0	35.4						
replicate	32.1	40.9	35.2						
replicate	34.5	30.3	37.1						
Mean	**34.0**	**35.8**	**35.2**	**43.6**	**39.8**	**38.2**	**35.8**	**40.2**	**40.1**
SD [cp/µL]	**2.2**	**2.8**	**3.1**	**7.4**	**3.2**	**5.1**	**4.0**	**3.6**	**6.1**

Source: JRC

Figure 6.2. Characterisation data for the ERM-AD482kb, the continuous line represents the certified value, the set of dashed lines reflects the certified range and the error bars represent the standard deviation of the measurements.



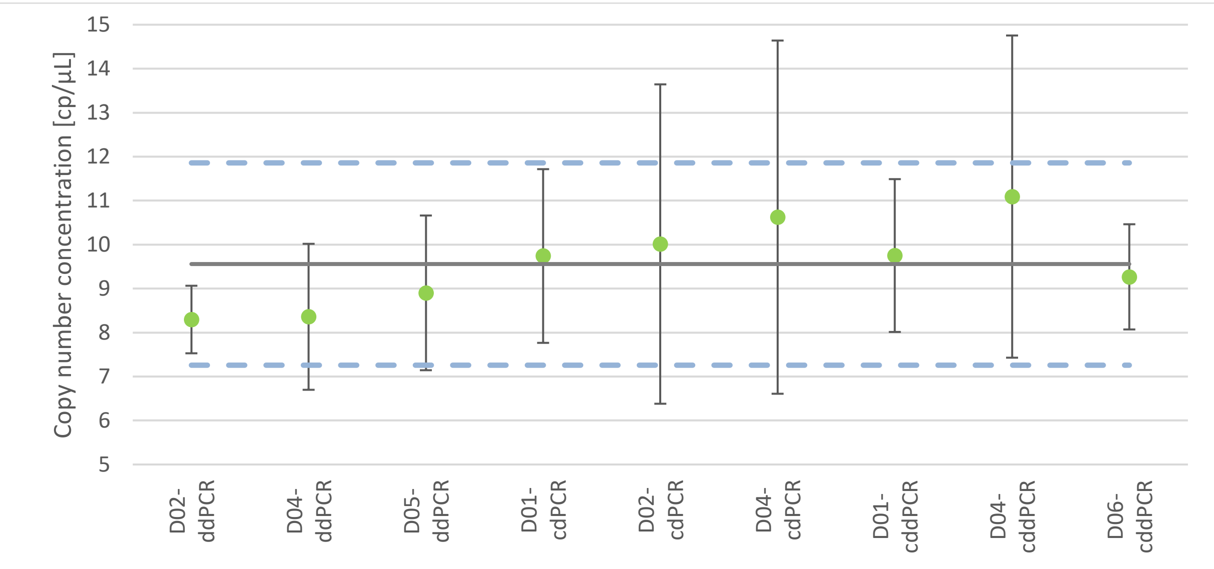
Source: JRC

Table 6.3. Individual results of the characterisation measurements of ERM-AD482kc.

Dataset code / Replicates [cp/µL]	D02-ddPCR	D04-ddPCR	D05-ddPCR	D01-cdPCR	D02-cdPCR	D04-cdPCR	LD1-cddPCR	D04-cddPCR	D06-cddPCR
replicate 1	7.5	6.8	7.0	10.0	5.2	10.0	6.4	6.7	10.1
replicate 2	8.9	6.8	9.7	10.0	8.0	20.1	9.3	11.3	8.8
replicate 3	9.0	11.0	7.8	10.1	13.2	7.2	13.2	8.8	10.3
replicate 4	7.3	7.0	9.0	6.7	17.3	12.9	11.1	11.1	7.9
replicate 5	9.2	10.3	14.4	10.4	9.2	7.9	9.1	9.2	11.0
replicate 6	8.0	6.8	9.8	6.0	7.7	10.3	10.7	15.8	10.4
replicate 7	8.1	10.3	8.7	11.6	13.4	13.3	9.8	8.2	8.8
replicate 8	*	8.5	10.0	9.2	9.4	9.6	8.9	10.6	9.7
replicate 9	8.0	10.0	11.0	11.5	6.9	8.2	9.4	18.9	7.6
replicate	8.0	6.2	6.8	11.8	9.8	6.7	9.6	10.3	8.0
replicate	8.5	8.2	7.3						
replicate	7.4	7.9	8.7						
replicate	8.3	7.6	10.6						
replicate	7.1	6.6	8.9						
replicate	9.0	7.9	8.3						
replicate	10.0	10.9	8.5						
replicate	8.7	6.2	8.0						
replicate	7.8	8.9	7.6						
replicate	9.2	8.6	7.0						
replicate	7.7	10.7	9.1						
Mean	**8.3**	**8.4**	**8.9**	**9.7**	**10.0**	**10.6**	**9.8**	**11.1**	**9.3**
SD [cp/µL]	**0.8**	**1.7**	**1.8**	**2.0**	**3.6**	**4.0**	**1.7**	**3.7**	**1.2**
*: measurement result excluded from the study due to technical reasons.									

Source: JRC

Figure 6.3. Characterisation data for the ERM-AD482kc, the continuous line represents the certified value, the set of dashed lines reflects the certified range and the error bars represent the standard deviation of the measurements.



Source: JRC

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