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CERTIFICATION REPORT

The certification of the copy number concentration of a poultry (chicken and turkey) plasmid DNA in solution: ERM®-AD484ka, ERM®-AD484kb, ERM®-AD484kc

EUR 30928 EN

European Commission
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Abstract
This report describes the production of ERM-AD484ka, ERM-AD484kb and ERM-AD484kc, which is a set of materials certified for the copy number concentration of a poultry (chicken and turkey) plasmid DNA. These materials were produced and certified in accordance with ISO 17034:2016 and ISO Guide 35:2017. ERM-AD484ka, ERM-AD484kb and ERM-AD484kc were produced within the scope of ISO 17034 accreditation of the JRC Geel.
A DNA fragment specific for the identification of a poultry (chicken and turkey) target was cloned into a pUC18 vector to construct the poultry plasmid. The nucleic acid sequence of the entire poultry plasmid was determined by dye terminator cycle sequencing. The plasmid was eluted in TElow buffer, its concentration was measured by ultraviolet (UV) spectrophotometry and it was gravimetrically diluted to three different concentration levels. The copy number concentration of the three concentration levels was certified by chamber digital quantitative polymerase chain reaction (cdPCR) and droplet digital PCR (ddPCR) methods. The CRM is available as a set of three polypropylene vials, each containing at least 1 mL of plasmid solution at one of the three copy number concentrations.
Between-unit homogeneity was quantified and stability during dispatch and storage was assessed in accordance with ISO Guide 35:2017. Within-unit homogeneity was quantified to determine the minimum sample size. The minimum sample size for one measurement is 3.9 µL.
The material was characterised by an interlaboratory comparison of laboratories of demonstrated competence and adhering to ISO/IEC 17025:2017. Technically invalid results were removed but no outlier was eliminated unless a technical reason for the deviation was found.
Uncertainties of the certified values were calculated in accordance with ISO 17034:2016 and ISO Guide 35:2017and include uncertainties related to possible inhomogeneity, instability and characterisation.
The material is only intended to be used for the determination of a cut-off value to discriminate positive samples (containing the target sequence) from negative samples by quantitative PCR (qPCR) as defined in the standard operating procedure of the European Union Reference Laboratory for the detection of Animal Proteins in feedingstuffs (EURL-AP) according to Commission Regulation (EU) No 51/2013.
As with any reference material, it can be used for establishing control charts. Before release of the CRM, the certification project was subjected to peer-review involving both internal and external experts.
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The certification of the copy number concentration of a poultry (chicken and turkey) plasmid DNA in solution: ERM®-AD484ka, ERM®-AD484kb, ERM®-AD484kc

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Disclaimer

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

Summary

This report describes the production of ERM-AD484ka, ERM-AD484kb and ERM-AD484kc, which is a set of materials certified for the copy number concentration of a poultry (chicken and turkey) plasmid DNA. These materials were produced and certified in accordance with ISO 17034:2016 [1] and ISO Guide 35:2017 [2]. ERM-AD484ka, ERM-AD484kb and ERM-AD484kc were produced within the scope of ISO 17034 accreditation of the JRC Geel.

A DNA fragment specific for the identification of a poultry (chicken and turkey) target was cloned into a pUC18 vector to construct the poultry plasmid. The nucleic acid sequence of the entire poultry plasmid was determined by dye terminator cycle sequencing. The plasmid was eluted in TE_{low} buffer, its concentration was measured by ultraviolet (UV) spectrophotometry and it was gravimetrically diluted to three different concentration levels. The copy number concentration of the three concentration levels was certified by chamber digital quantitative polymerase chain reaction (cdPCR) and droplet digital PCR (ddPCR) methods.

The CRM is available as a set of three polypropylene vials, each containing at least 1 mL of plasmid solution at one of the three copy number concentrations.

Between-unit homogeneity was quantified and stability during dispatch and storage was assessed in accordance with ISO Guide 35:2017 [2]. Within-unit homogeneity was quantified to determine the minimum sample size. The minimum sample size for one measurement is 3.9 µL.

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 were calculated in accordance with ISO 17034:2016 [1] and ISO Guide 35:2017 [2] and include uncertainties related to possible inhomogeneity, instability and characterisation.

The material is only intended to be used for the determination of a cut-off value to discriminate positive samples (containing the target sequence) from negative samples by quantitative PCR (qPCR) as defined in the standard operating procedure of the European Union Reference Laboratory for the detection of Animal Proteins in feedingstuffs (EURL-AP) according to Commission Regulation (EU) No 51/2013 [4] [5].

As with any reference material, it can be used for establishing control charts.

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

The following values were assigned:

Copy number concentration[1)]		
	Certified value [2)] [cp/µL]	Uncertainty [3)] [cp/µL]
ERM-AD484ka	125	12
ERM-AD484kb	33	4
ERM-AD484kc	7.7	1.7

[1)] As obtained by chamber digital PCR (cdPCR) and droplet digital PCR (ddPCR) methods using the poultry (chicken and turkey) specific primers and probe described in the standard operating procedure developed by the European Union Reference Laboratory for Animal Proteins in feedingstuffs [EURL-AP-SOP-Poultry-PCR-V1.0.pdf (craw.eu)]

[2)] Certified values are values that fulfil the highest standards of accuracy. The given value(s) represent(s) 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 Guide 35:2017.

Table of contents

Glossary

ANOVA	Analysis of variance
b	Slope in the equation of linear regression $y = a + bx$
bp	Base pair
c_{DNA}	DNA mass concentration
cdPCR	Chamber 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	Ethylenediaminetetraacetic acid
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
LN	Liquid Nitrogen
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

p	Number of technically valid datasets
PAP	Processed animal protein
PCR	Polymerase chain reaction
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 ti
t_i	Time point for each replicate
t_{sl}	Chosen shelf life
t_{tt}	Chosen transport time
TE	Buffer containing TRIS and EDTA
TE_{low}	Buffer containing TRIS, 0.01 mmol/L EDTA at pH 8.0
TRIS	Tris(hydroxymethyl)aminomethane
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
$\bar{x}$	Arithmetic mean
Δ_{meas}	Absolute difference between mean measured value and the certified value
$\nu_{MSwithin}$	Degrees of freedom of MS_{within}
$\bar{y}$	Mean of all results of the study

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) banning all PAPs from feed and banning intra-species recycling [6] [7] [8]. In 2013, the ban of non-ruminant PAPs to be used in aquacultures was lifted [9] and in 2021, the ban of non-ruminant PAPs to be used in the feed of non-ruminant animals was lifted through Regulation (EU) 2021/1372, maintaining the ban on intra-species recycling [10].

Following these regulations, methods for species-specific detection of PAPs in compound feeds are required. Regulation (EC) No 152/2009 lays down the methods of sampling and analysis for the official control of feed [11]. Regulation (EU) No 51/2013 includes PCR-based methods for the detection of animal constituents [5]. Using qPCR methods, it is possible 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.

qPCR methods for the detection of animal DNA in feed-stuff have been validated by the EURL-AP. Calibrants are needed to enable the implementation of these qPCR protocols used as an official control method for detecting the presence of PAPs in feed. In particular, these calibrants are used to determine a threshold or cut-off value at which the PCR signal is considered 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 [12]. Therefore, each control laboratory must determine its own instrument- and method-specific threshold value and verify this value on a regular basis.

In the light of these reauthorisations, certified reference materials (CRMs) are required. JRC has produced CRMs for the detection of PAPs of ruminant (ERM-AD482 series), porcine (ERM-AD483 series) and now, with the production of the ERM-AD484k series, of poultry origin, to set the cut-off value of the respective qPCR methods.

1.2 Choice of the material

The ERM-AD484k series, with concentration levels a, b and c consist of linearised plasmid DNA in buffered solution certified for their absolute DNA copy number concentration to allow control laboratories to determine their cut-off values for the poultry assay [4, 12] and to report their results in line with Regulation (EU) No 51/2013 [5].

The plasmid of ERM-AD484k, contains a poultry (chicken and turkey) specific mitochondrial target DNA fragment of 84 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 buffered plasmid solution is stabilised using Salmon Testes DNA as background DNA. The material is a liquid frozen material. The target concentration levels are set as defined by the standard operating procedure for the detection of poultry DNA in feed using real-time PCR [4], validated by the EURL-AP.

1.3 Outline of the CRM project

The production of a CRM as defined in ISO 17034 [1] is a project comprising planning, processing of the material, homogeneity and stability testing, characterisation and assigning of the property values and finally distribution and post-certification monitoring to control stability.

An *Escherichia coli* (*E. coli*) strain bearing a pUC18 plasmid containing 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 measured by UV spectrophotometry and digital droplet PCR and diluted gravimetrically to obtain three solutions at target concentrations of 128 cp/µL, 32 cp/µL and 8 cp/µL, which were dispensed in polypropylene tubes.

Homogeneity and stability, including freeze-thaw stability, were assessed by ddPCR. Confirmation of the identity was done by DNA sequencing and characterisation of the copy number concentration was done by an interlaboratory comparison applying cdPCR and ddPCR.

Uncertainties of certified values were estimated in compliance with ISO 17034 [1], which implements the basic principles of ISO/IEC Guide 98 (GUM) [13].

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.

2 Participants

2.1 Project management and data evaluation

European Commission, Joint Research Centre, Directorate F – Health, Consumers and Reference Materials, Unit F.6 (Reference Materials Unit), Geel, BE
(accredited to ISO 17034:2016 for production of certified reference materials, BELAC No. 268-RM)

2.2 Provider of raw material and quantification method

The EURL-AP provided the raw material, being the poultry plasmid, and the quantitative PCR method for the detection of poultry (chicken and turkey) DNA in feed which was validated and published in September 2021 by the EURL-AP.

2.3 Processing

European Commission, Joint Research Centre, Directorate F – Health, Consumers and Reference Materials, Unit F.6 (Reference Materials Unit), Geel, BE
(accredited to ISO 17034:2016 for production of certified reference materials, BELAC No. 268-RM)

Baseclear BV, Leiden, NL

2.4 Homogeneity measurements

European Commission, Joint Research Centre, Directorate F – Health, Consumers and Reference Materials, Geel, BE

2.5 Stability measurements

European Commission, Joint Research Centre, Directorate F – Health, Consumers and Reference Materials, Geel, BE

2.6 Characterisation measurements

European Commission, Joint Research Centre, Directorate F – Health, Consumers and Reference Materials, 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, SE

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

All laboratories are identified by a code (e.g. L01). The numbering does not follow the alphabetical order presented above.

3 Material processing and process control

3.1 Origin of of the starting material

The plasmid of interest was constructed by cloning a poultry (chicken and turkey) specific mitochondrial DNA fragment of 84 bp into a pUC18 vector by the EURL-AP and provided to the JRC as a bacterial culture in Luria Bertani (LB) medium supplemented with glycerol.

3.2 Processing

The bacterial culture bearing the plasmid of interest was cultured on LB medium 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 (QIAprep Spin Miniprep Kit, Qiagen) and suspended in sterile TE_{low} buffer (1 mM Tris, 0.01 mM EDTA, pH 8). 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), suspended in sterile TE_{low} buffer and kept at 4 °C.

The purified plasmid solution was diluted in sterile TE_{low} containing a background of Salmon Testes DNA (50 ng/µL) (*Oncorhynchus keta*, Sigma Aldrich Cat. No.: D-1626) to prepare a stock solution. From this stock solution, three independent dilutions were gravimetrically prepared to obtain 2 L of solution, each with the following target concentrations: 128 cp/µL, 32 cp/µL and 8 cp/µL. The solutions were kept at 4 °C until further processing.

From each final solution, a volume of approximately 1.1 mL was dispensed into 1400 sterile pre-labelled, low binding polypropylene vials (Axygen, cat. No. SCT-150-SS-L-S) under sterile and cooled conditions. Once filled, all vials were manually closed and were stored at 4 °C. Once all vials were filled, they were shock frozen in liquid nitrogen (LN) and stored at -70 °C.

Sets of the plasmid solutions were prepared by assembling one vial of each concentration level into a plastic box after which sets were stored at -20 °C. At no point after the initial freezing were the vials allowed to thaw.

For the purpose of this report, the term 'unit' refers to one vial of ERM-AD484ka, ERM-AD484kb or ERM-AD484kc.

3.3 Process control

3.3.1 Plasmid size confirmation

Completeness of the enzymatic digestion and of the size of the plasmid was confirmed by agarose gel electrophoresis.

3.3.2 Plasmid identity confirmation

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

The sequence of the plasmid was determined by double stranded Sanger sequencing of the entire circular plasmid, applying the primer walking method with a minimum two fold coverage.

Sequencing was performed by Baseclear 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 dideoxy terminator 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 during processing and performed double stranded sequencing of the entire circular plasmid by primer walking method. Sequences were generated using the BigDye® v3.1 chemistry (Life Technologies). The sequence reactions were analysed on an ABI3730 sequencer (Genetic analyser, Life Technologies). A full sequence of the plasmid was provided.

Sequences were aligned using the Basic Local Alignment search Tool (BLAST) [14]. Alignment of the obtained sequence with the reference sequence, provided by the EURL-AP, confirmed a 99.96 % match between the sequences with one single mismatch between the sequences. The single mismatch was located in the fragment originating from the pUC18 vector and has therefore no effect on the suitability of the reference material. Result of the alignment can be found in Annex A.

3.3.3 Purity of the plasmid extract

Different types of contaminants may be present in the plasmid extract: plasmids used for the assembly of the plasmid, nucleic acids originating from host bacterial cells and non-nucleic acid contaminants.

During the cloning process, the bacterial cells could have been transformed with different populations of plasmids. The synthetic plasmids used for the cloning (pUC18 and pCR 2.1) have the same origin of replication (*ori*V 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 [15]. 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.

After linearisation by enzymatic digestion with *Ssp*I restriction enzyme and purification with the Qiaquick® Gel extraction kit, the purified plasmid solution was analysed by gel electrophoresis. As no smear and/or RNA bands were observed, 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 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 260 nm (A260) and 280 nm (A280), where pure nucleic acid samples are expected to have a theoretical A260/A280 ratio of approximately 1.8 [16]. The A260/A280 was measured as 1.89, 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 used are highly specific for the targeted sequences and do not hybridise with other DNA fragments, which could be present in the final plasmid preparation.

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

Calculation of the molar mass of the linear plasmid was based on the length of the plasmid and an average molar mass of 615 g/mol per base pair [17].

Nanodrop spectrophotometry measured the plasmid mass concentration of the stock solution as 24.17 ng/µl, resulting in a copy number concentration of 8.28 x 10^9 cp/µl. This value was used to calculate the dilution needed to prepare the stock solution.

3.3.4 Concentration verification

The copy number concentrations of the stock solution and of the diluted plasmid solutions were verified by ddPCR. The measured copy number concentrations were in close agreement with the target concentrations (data not shown).

4 Homogeneity

A key requirement for any reference material produced as a batch of units is equivalence between those units. In this respect, it is relevant whether the variation between units is significant compared to the uncertainty of the certified value, it however is not relevant whether this variation between units is significant compared to the variation of measurement results. Consequently, ISO 17034 [1] requires RM producers to quantify the between-unit variation. This aspect is covered in between-unit homogeneity studies.

The within-unit inhomogeneity does not influence the uncertainty of the certified value when the minimum sample 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 was evaluated to ensure that the certified value of the CRM is valid for all units of the material, within the stated uncertainty.

The number of units selected corresponds to approximately the cube root of the total number of units produced for each concentration level. Twelve units were selected using a random stratified sampling scheme covering the whole batch for the between-unit homogeneity test. Random stratified sampling involves dividing the batch into twelve groups (with a similar number of units in each group) and selecting one unit randomly from each group. Six independent samples were taken from each selected unit, and 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.

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 were observed at a 95 % confidence level. A significant (95 % confidence level) trend in the measurement sequence was observed for ERM-AD484ka, pointing at a changing parameter, e.g. a signal drift in the measurement system. Research has shown that the correction of even non-significant biases is beneficial. Such correction of non-significant biases has two advantages: on the one hand, the resulting uncertainties are smaller than for non-corrected biases. On the other hand, after correction the uncertainty interval has the highest probability of including the true value [18]. Correction of trends is therefore expected to improve the sensitivity of subsequent statistical analysis through a reduction in the variation of measurement results without masking potential between-unit heterogeneities. As the measurement sequence and the unit numbers were not correlated, trends significant on at least a 95 % confidence level were corrected as shown below:

$$x_{i\_corr} = x_i - b \cdot i \qquad \textbf{Equation 2}$$

b	slope of the linear regression
i	position of the result in the measurement sequence

The datasets, the trend-corrected dataset in the case of ERM-AD484ka, were assessed for consistency using Grubbs outlier tests at a confidence level of 99 % on the individual results and on the unit means. No outlying individual results and outlying unit means were detected.

Quantification of between-unit inhomogeneity 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, which follow at least a unimodal distribution and results for each unit that follow unimodal distributions with approximately the same standard deviations. The distribution of individual values and 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-AD484ka	Yes (corrected)	no	none	none	unimodal	normal
ERM-AD484kb	no	no	none	none	unimodal	normal
ERM-AD484kc	no	no	none	none	normal	normal

[1] 95 % confidence level
[2] 99 % confidence level

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.* [19]. u^*_{bb} is comparable to the limit of detection (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:

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

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

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

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

Table 2: Results of the homogeneity studies

CRM-code	$s_{wb,\,rel}$ [%]	$s_{bb,\,rel}$ [%]	$u^*_{bb,\,rel}$ [%]	$u_{bb,\,rel}$ [%]
ERM-AD484ka	5.81	1.52	1.01	1.52
ERM-AD484kb	8.59	n.c.[1)]	1.61	1.61
ERM-AD484kc	18.28	8.92	3.42	8.92

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

The homogeneity studies showed no outlying unit means or trends in the filling sequence. Therefore, the between-unit standard deviation can be used as an 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.

4.2 Within-unit homogeneity and minimum sample size

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

The minimum sample size was not established experimentally but based on the technically valid results of the characterisation study, using the method information supplied by the participants. The smallest sample intake that still yielded results with acceptable precision to be included in the respective study was taken as minimum sample size. Based on the characterisation data from Annex G, a minimum sample size of 3.9 µL is derived.

5 Stability

Time and temperature were regarded as the most relevant influences on the stability of the material. Therefore, the influences of time and temperature needed to be investigated.

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

The stability studies were carried out using an inversed isochronous design, closely related to a normal isochronous design [20]. In this approach, units were stored for a particular length of time under conditions where degradation can be assumed negligible (reference conditions). Afterwards, the units were moved to different temperature conditions. At the end of the inversed isochronous storage, the samples were analysed simultaneously under repeatability or intermediate precision conditions. Analysis of the material (after various exposure times and temperatures) under repeatability or intermediate precision conditions greatly improves the sensitivity of the stability tests.

5.1 Short-term stability study

In the short-term stability study, the conditions for dispatch of the material to the customers were established. To this end, units were stored at -20 °C and 4 °C for 0, 1, 2, 3 and 4 weeks (at each temperature). 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 intermediate precision conditions (spread over two 96-well plates due to size limitations) and in a random sequence to be able to separate any potential drift in the measurement results or any plate effects from a potential trend over storage time.

The data were evaluated individually for each temperature. Results were screened for outliers using the single and double Grubbs test on a confidence level of 99 % and differences between plates were checked using a *t*-test at a 95 % confidence level.

In addition, the data were evaluated against storage time, and regression lines of copy number concentration versus time were calculated, to test for potential increases or decreases of the 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 D. The results of the statistical evaluation of the short-term stability are summarised in Table 3.

Table 3: Results of the short-term stability tests

CRM-code	Number of individual outlying results [1)]		Significance of the trend [2)]	
	-20 °C	4 °C	-20 °C	4 °C
ERM-AD484ka	none	none	yes (positive)	yes (negative)
ERM-AD484kb	none	none	no	no
ERM-AD484kc	none	none	no	no

[1)] 99 % confidence level

[2)] 95 % confidence level

As the measurements were performed under intermediate precision conditions (spread over two plates), it had to be checked if there were significant differences between the plate means using a *t*-test as plate effects can occur that could mask a potential trend. No significant plate effects were observed at a confidence level of 95 % for any of the concentrations.

For ERM-AD484ka a significant trend in the measurement sequence was observed at 95 % confidence level, pointing at instability of the analytical system. This trend was corrected before further data analysis.

No outliers were detected and all data were retained for the estimation of u_{sts}. For ERM-AD484kb and c, none of the trends were statistically significant on a 95 % confidence level for any of the temperatures. For ERM-AD484ka a significant trend at +4 °C was found, indicating a degradation at this temperature. A significant trend was also found at -20 °C, this was however a positive trend. Further investigation showed that the trend could be attributed to a shift between the reference temperature and the test temperature values rather than a gradual increase over time. This leads to the conclusion that although additional copies of the target DNA cannot be created in the sample, thawing the samples directly from -70 °C can affect the accessibility of the target DNA fragment for the primers and probe, resulting in lower copy number concentrations measured, compared to samples thawed from -20 °C.

As a result of the outcome of the short-term stability study, samples stored at -70 °C were moved to -20 °C for at least one week before analysis.

The material shall be shipped frozen on dry ice.

5.2 Long-term stability study

Long-term storage conditions and shelf life guaranteeing the stability of the material and the certified values were established.

Data from two inversed isochronous stability studies were combined to assess the stability of the CRM.

For the first inversed isochronous study, units were stored at -20 °C for 0, 8, 11, 14 and 17 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 intermediate precision conditions (spread over two 96-well plates due to size limitations) and in a random sequence to be able to separate any potential drift in the measurement results or any plate effect from a potential trend over storage time. A normalisation was applied when a significant plate effect was present

For the second inversed isochronous study, units were stored at -20 °C for 0, 18 and 24 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 for each study.

As the measurements of the 17 month study were performed under intermediate precision conditions (spread over two plates), it had to be checked if there were significant differences between the plate means using a *t*-test as plate effects can occur that could mask a potential trend. No significant plate effects were observed at a confidence level of 95 %.

For ERM-AD484kb and ERM-AD484kc data from the 24 month study and for ERM-AD484kb data from the 17 month study a significant trend in the measurement sequence was observed at 95 % confidence level, pointing at instability of the analytical system. These trends were corrected before further data analysis.

The results were screened for outliers using the single and double Grubbs test at a confidence level of 99 %. No statistical outliers were detected, and the results were retained for the estimation of u_{lts}.

Furthermore, the data were plotted against storage time, and regression lines of copy number concentration versus time were calculated. The slope of the regression lines was tested for statistical significance (loss/increase due to storage). For ERM-AD484ka and ERM-AD484kb, the slopes of the regression lines were significantly different from zero (95 % confidence level).

Afterwards the results of the two inversed isochronous studies were combined as described in [21]. For ERM-AD484ka a measurement bias between the two studies was found and corrected using normalisation factor *d* as calculated in the following equation:

$$d = \bar{x}_2 / \bar{x}_1 \qquad \textbf{Equation 6}$$

$\bar{x}_1$ mean measurement result of study 1
$\bar{x}_2$ mean measurement result of study 2

The relative uncertainty of this correction $u_{d, rel}$ was calculated as:

$$u_{d, rel} = \sqrt{\frac{1}{n_1} RSD_1^2 + \frac{1}{n_2} RSD_2^2} \qquad \textbf{Equation 7}$$

RSD_1: relative standard deviation of all results in study 1
RSD_2: relative standard deviation of all results in study 2
n_1: number of data points in study 1
n_2: number of data points in study 2

For ERM-AD484ka the uncertainty related to the correction of the measurement bias between the long-term stability studies, $u_{d, rel}$ is 1.20 %.

Again, the combined data were plotted against storage time and regression lines of copy number concentration versus time were calculated. The slopes of the regression lines were tested for statistical significance. For ERM-AD484ka and b the slopes of the regression lines were significantly different from zero (95 % confidence level), also here a positive trend was observed. Further investigation showed that the trend could still be attributed to a shift between the reference temperature and the test temperature values, rather than a gradual increase over time, even though the reference samples were moved to -20 °C one week before analysis. In order to account for the trend, it will be taken up in the calculation of the uncertainty contribution related to stability during storage of ERM-AD484ka and b.

The results of the measurements are shown in Annex E.

The material will be stored at -20 °C.

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

5.3 Repeated use stability studies

For ERM-AD484k, the impact of repeated use and stability upon thawing has been investigated.

To this end a study to investigate the resistance of the material to freeze-thaw cycles and a dedicated short-term stability study were performed.

The freeze-thaw study was performed before processing of ERM-AD484ka, b and c, using three pilot batches with the same target concentrations. For the freeze-thaw study, two units of the pilot batches were subjected to a freeze-thaw cycle at -20 °C and two units were not frozen. From each unit two samples were measured by ddPCR. A decrease in copy number concentration was observed after one freeze-thaw cycle at -20 °C, most pronounced for the highest concentration. Further investigation, applying the same experimental setup to units frozen at -70 °C and in LN showed that the decrease was less pronounced after a freeze-thaw cycle at -70 °C and no significant decrease was observed after a freeze-thaw cycle in LN. It was therefore decided to freeze ERM-AD484ka, b and c in LN after production, with subsequent storage at -20 °C, and to instruct users to avoid freezing after initial thawing.

The characterisation study revealed that while ddPCR measurements remained unaffected, and cdPCR measurements performed immediately after thawing appeared normal, amplification problems occurred when applying cdPCR to units thawed for longer than 1 day. To investigate this further, a dedicated short-term stability study was performed on ERM-AD484ka and ERM-AD484kb, applying cdPCR and qPCR. The stability study was carried out using a classical design. In this setup units were measured 2, 5 and 8 hours after thawing alongside freshly thawed units at each time point and analysed by cdPCR and qPCR. Two units per storage time were selected. From each unit two samples were measured by cdPCR and three samples were measured by qPCR. Additional qPCR measurements were performed on two units of ERM-AD484ka, 22 and 27 hours after thawing. Due to the classical stability study setup, the measurements were performed under intermediate precision conditions.

The study confirmed the outcome of the characterisation study with no problems of amplification within 8 hours after thawing but reduced amplification with samples measured for longer after initial thawing. It was therefore decided to instruct users to use the material within 8 hours after thawing.

Results of the measurements are shown in Annex F.

5.4 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 dispatch were estimated, as described in [22]. In this approach, the uncertainty of the linear regression line with a slope of zero was calculated. The uncertainty contribution u_{sts} was calculated as the product of the chosen transport time and the uncertainty of the regression lines as:

$$u_{sts,\,rel} = \frac{s_{rel}}{\sqrt{\sum (t_i - \bar{t})^2}} \cdot t_{tt} \qquad \textbf{Equation 8}$$

s_{rel} relative standard deviation of all results of the stability study
t_i time elapsed at time point *i*
$\bar{t}$ mean of all t_i
t_{tt} chosen transport time (1 week at -20 °C)

The uncertainties of stability during storage of ERM-AD484ka and ERM-AD484kb were estimated, taking into account the trend observed in the long-term stability study. To achieve this, the uncertainty of the linear regression line, with a slope reflecting the trends observed, was calculated. The

uncertainty contribution $u_{lts,rel,}$ was calculated as the product of the chosen shelf life and the uncertainty of the regression lines as:

$$u_{lts,\,rel} = \frac{\sqrt{(\frac{b}{\sqrt{3}})^2 + s_b^2}}{\bar{y}} \cdot t_{sl}$$ **Equation 9**

b slope of the linear regression
$\bar{y}$ mean of all results of the stability study
s_b standard deviation of the slope
t_{sl} chosen shelf life (12 months at -20 °C)

The uncertainty of stability during storage of ERM-AD484kc was estimated in the same way as for stability during dispatch. The uncertainty of the linear regression line with a slope of zero was calculated and the uncertainty contribution u_{lts} was calculated as the product of the chosen shelf life and the uncertainty of the regression line as:

$$u_{lts,\,rel} = \frac{s_{rel}}{\sqrt{\sum (t_i - \bar{t})^2}} \cdot t_{sl}$$ **Equation 10**

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

The following uncertainties were estimated:

- $u_{sts,rel}$, the uncertainty of stability during dispatch. This was estimated from the -20 °C studies. The uncertainty describes the possible change during a dispatch at -20 °C lasting for one week.
- $u_{lts,rel}$, the uncertainty of stability during storage. This was estimated from the -20 °C studies. The uncertainty contribution describes the possible degradation during storage for 12 months at -20 °C.
- $u_{d,rel}$, the uncertainty of the correction of the measurement bias between two long-term stability studies.

The results of these evaluations are summarised in Table 4.

Table 4: Uncertainties of stability during dispatch 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	$u_{sts,rel}$ [%]	$u_{lts,rel}$ [%]	$u_{d,rel}$ [%]
ERM-AD484ka	0.52	3.22	1.20
ERM-AD484kb	0.71	3.19	n.a.[1)]
ERM-AD484kc	1.23	2.37	n.a.[1)]

[1)] n.a.: not applicable as no measurement bias correction needed

6 Characterisation

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

This was based on an interlaboratory comparison of expert laboratories, i.e. the copy number concentration of the material was determined in different laboratories that applied different measurement procedures to demonstrate the absence of a measurement bias. Due to the nature of the measurand all participants used a similar method for the measurements. This approach converts the systematic bias of each laboratory into a random variable, the combined effect of which is reduced by averaging over several laboratories.

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 dPCR measurements in relevant matrices by submitting results for 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

Each laboratory received two units of each concentration level of ERM-AD484k and was requested to provide 16 independent results, 8 per unit, for ddPCR and 10 independent results, 5 per unit, for cdPCR. The units for material characterisation were selected using a random stratified sampling scheme and covered the whole batch. In the case of ddPCR, measurements were spread over two 96-well plates to ensure intermediate precision conditions. In the case of cdPCR, measurements were spread over three digital arrays, however the two units of each concentration were measured on the same digital array.

Laboratories were also requested to give estimations of the expanded uncertainties of the mean value of the results. No approach for the estimation was prescribed, i.e. top-down and bottom-up [13] were regarded as equally valid procedures.

6.3 Methods and measurement procedures used

Two different dPCR methods, both without sample preparation, with different quantification steps were used to characterise the material. The combination of results from the methods both targeting the same measurand but based on different measurement principles mitigates undetected method bias.

The methods used during the characterisation study are described in Annex B and listed in Annex G. The laboratory code (e.g. L01-ddPCR) is a random number and does not correspond to the order of laboratories in Section 2. The lab-method code consists of a number assigned to each laboratory (e.g. L01) and abbreviation of the measurement method used (e.g. ddPCR).

All laboratories used dPCR for determination of the copy number concentration, part of the laboratories applied a cdPCR method while other labs applied a ddPCR method and some laboratories provided data for both methods.

6.4 Evaluation of results

The characterisation study resulted in eight datasets per concentration. All individual results of the participating laboratories, grouped per concentration, are displayed in tabular and graphical form in Annex G.

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 was considered during the evaluation:

- Compliance with the instructions given: 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.

The characterisation data revealed that the results obtained by cdPCR were affected by the amount of time the samples were analysed after thawing. Lower copy number concentrations were measured in samples, which were thawed for more than 1 day before performance of cdPCR measurements. A dedicated short-term stability investigation (Section 5.3) confirmed this, which led to the conclusion that only results of samples measured within 1 day after thawing were considered technically valid.

Based on the above criteria and the additional findings, the following datasets were rejected as not technically valid (Table 5). No data was rejected for non-compliance with the instructions, several datasets were rejected for being measured to long after thawing.

Table 5: Datasets that were rejected as not technically valid and action taken

CRM-code	Lab-method code	Description of problem	Action taken
ERM-AD484ka	L02 - cdPCR	Measured >1 day after thawing	not used for evaluation
	L03 - cdPCR	Measured >1 day after thawing	not used for evaluation
ERM-AD484kb	L02 - cdPCR	Measured >1 day after thawing	not used for evaluation
	L03 - cdPCR	Measured >1 day after thawing	not used for evaluation
ERM-AD484kc	L02 - ddPCR	Measured >1 day after thawing	not used for evaluation

6.4.2 Statistical evaluation

The datasets accepted based on technical reasons were tested for normality of dataset means using kurtosis/skewness tests and, in the case of too few datasets, tested visually using histograms and normal probability plots. Accepted datasets were further 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.

Table 6: Statistical evaluation of the technically accepted datasets for ERM-AD484k with *p* being the number of technically valid datasets

CRM-code	*p*	Outliers		Normally distributed	Statistical parameters			
		Means	Variances		Mean [cp/µL]	*s* [cp/µL]	$s_{between}$ [cp/µL]	s_{within} [cp/µL]
ERM-AD484ka	6	no	no	yes	124.66	8.11	7.05	8.78
ERM-AD484kb	6	no	no	yes	32.63	3.19	2.63	3.49
ERM-AD484kc	7	no	no	yes	7.65	0.99	0.84	1.67

The laboratory means follow normal distributions. None of the data contains outlying means and variances. Considering the use of the CRM to set the cut-off value of a qPCR method, the datasets are in sufficient agreement and the mean of laboratory means is a good estimate of the true value.

The uncertainty related to the characterisation is estimated as the standard error of the mean of laboratory means ($s/\sqrt{p}$). (Table 7).

Table 7: Uncertainty of characterisation for ERM-AD484k

CRM-code	*p*	Mean [cp/µL]	*s* [cp/µL]	u_{char} [cp/µL]
ERM-AD484ka	6	124.66	8.11	3.31
ERM-AD484kb	6	32.63	3.19	1.30
ERM-AD484kc	7	7.65	0.99	0.38

7 Value Assignment

Certified values were assigned.

Certified values are values that fulfil the highest standards of accuracy. Procedures at JRC Directorate F recommend pooling of at least six datasets to assign certified values. Full uncertainty budgets in accordance with ISO 17034 [1] and ISO Guide 35 [2] were established.

7.1 Certified values and their uncertainties

The unweighted mean of the means of the accepted datasets as shown in Table 6 was assigned as certified value for each parameter.

The assigned uncertainties 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}), including the correction of the measurement bias between two stability studies ($u_{d,rel}$) when applicable. 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:

$$U_{CRM,\,rel} = k \cdot \sqrt{u^2_{bb,\,rel} + u^2_{sts,\,rel} + u^2_{lts,\,rel} + u^2_{d,\,rel} + u^2_{char,\,rel}} \qquad \textbf{Equation 11}$$

- u_{char} was estimated as described in Section 6.4
- u_{bb} was estimated as described in Section 4.1
- u_{sts} , u_{lts} and $u_{d,rel}$ were estimated as described in Section 5.1 and 5.2

The JRC's procedures for assigning uncertainties to certified values stipulate that 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 8, the $u_{char,\,rel}$ and $u_{lts,\,rel}$ are the dominant contributions to the combined uncertainty. From these $u_{char,\,rel}$ has the least amounts of freedom but still 5 degrees of freedom for ERM-AD484ka and ERM-AD484kb and 6 degrees of freedom for ERM-AD484kc. Therefore, a k-factor of 2 was applied to obtain the expanded uncertainties.

The certified values and their uncertainties are summarised in Table 8.

Table 8: Certified values and their uncertainties for ERM-AD484k.

CRM-code	Certified value [cp/µL]	$u_{char,\,rel}$ [%]	$u_{bb,\,rel}$ [%]	$u_{sts,\,rel}$ [%]	$u_{lts,\,rel}$ [%]	$u_{d,\,rel}$ [%]	$U_{CRM,\,rel}$ [%]	U_{CRM} [1)] [cp/µL]
ERM-AD484ka	125	2.66	1.52	0.52	3.22	1.20	9.26	12
ERM-AD484kb	33	3.99	1.61	0.71	3.19	n.a.[1)]	10.80	4
ERM-AD484kc	7.7	4.91	8.92	1.23	2.37	n.a. [1)]	21.05	1.7

[1)] n.a.: not applicable as no measurement bias correction needed

Expanded (k = 2) and rounded uncertainty; uncertainties are always rounded up [23] and in a way that the rounding error corresponds to 3 % to 30 % of the uncertainty.

8 Metrological traceability and commutability

8.1 Metrological traceability

Identity

Poultry (chicken and turkey) plasmid DNA is a (chemically) clearly defined analyte, its identity was confirmed by sequencing. The participants used different measurement principles for the quantification, demonstrating absence of measurement bias. Nevertheless, since all participants used methods based on digital PCR, the measurand is operationally defined as obtained by digital PCR, using the specific primers and probe shown in table B1.

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

The concept of commutability of a reference material is defined by the VIM [15] 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"

The structure of the poultry (chicken and turkey) plasmid DNA is clearly defined via its DNA sequence. The CRM was certified for its absolute copy number concentration with the aim to implement the detection method set in the corresponding EU legislation for PAPs in feed.

The CRMs are intended for setting the cut-off value of real-time PCR measurements of poultry (chicken and turkey) DNA in feed. Consequently in combination with the measurement method validated by the EURL-AP [4], this set of CRMs is establishing the arbitrary reference system required for the detection of poultry (chicken and turkey) 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

The material should be stored at -20 ± 5 °C prior to use.

The solutions need to be used within 8 hours after thawing and should not be re-frozen.

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.

9.4 Minimum sample size

The minimum sample size is 3.9 µL.

9.5 Use of the certified value

The intended use of this material 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-Poultry-PCR-V1.0.pdf (craw.eu)). The set should be used to construct calibration curves at low copy number concentration 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 poultry animal proteins from samples positive for poultry animal proteins.

As with any reference material, it can be used for establishing control charts.

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 [24]).

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 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.

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Annex A: 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.

Alignment information: Identities: 2856/2857(99 %), Gaps: 0/2857(0 %), 99.96 % overall identity.

Highlighted in blue: 84 bp poultry (chicken and turkey) specific mitochondrial DNA fragment, primer and probe annealing site indicated in bold and underlined. The single mismatch indicated in red (base 1196).

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Query  1     ATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTG  60
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Sbjct  61    CGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTG  120

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Sbjct  121   AAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCC  180

Query  181   TTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTAT  240
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Annex B: Description of the dPCR methods

Droplet digital PCR

The protocol described was used for all ddPCR measurements at JRC Geel, as well as by all the laboratories performing ddPCR measurements in the characterisation study. The sequences of primers and probes were provided by the EURL-AP. Volumes of 7.7 µL of the undiluted ERM-AD484ka, ERM-AD484kb, ERM-AD484kc 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 poultry (chicken and turkey) target at final concentrations as outlined in Table B1, together with 2x ddPCR Supermix for probes no dUTP reagent (Ref. 1863024) (11 µL) from Bio-Rad (Bio-Rad Laboratories N.V., Temse, BE). The amplification was performed according to the specifications in Table B2. After amplification the droplets were read and the results analysed with the QuantaSoft software using the appropriate threshold for each analysis.

Table B1: Primers and probes used to amplify the poultry (chicken and turkey) target

Primer/ probe	Sequence	Concentration in PCR reaction [nM]	Amplicon size [bp]
Forward primer	5'-TAG ACT ACC AAG GCG TAG CT-3'	600	84
Reverse primer	5'-AAG TCA AGG CGA CCT TG-3'	600	
Probe	FAM-5'- AAA GCA TTC AGC TTA CAC CTG AAA-3'-TAMRA	360	

Table B2: Thermal cycling protocol used for the simplex PCR reaction amplifying the poultry (chicken and poultry) 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	50	

Chamber digital PCR

The protocol described was used for all cdPCR measurements at JRC Geel, as well as by all the laboratories performing chamber dPCR measurements in the characterisation study. The sequences of primers and probes were provided by the EURL-AP.

Volumes of 3.9 µL of the undiluted ERM-AD484ka, ERM-AD484kb, ERM-AD484kc plasmid solutions were used in the dPCR assay. A volume of 3.9 µL of the DNA sample was mixed with 6.1 µL of Pre-sample mix solution and loaded into a well of the digital array primed with control line fluid. The 6.1 µL Pre-sample mix solution contains the primers and probes (0.2 µL each) for the poultry (chicken and turkey) target at final concentrations as outlined in Table B1, together with 2x Diagenode Universal Mastermix (Cat. No.: DMMLD2D600) (5 µL) from Diagenode (Diagenode sa. Belgium, Seraing (Ougrée), BE). The amplification was performed according to the specifications in Table B3, the image of the digital array was captured at the end of each cycle. For the automatic calling of the partitions, a quality threshold of 0.4, a linear baseline correction, auto (global) Ct threshold method and target Ct interval between 20 and 50 were used by the software settings.

Table B3: Thermal cycling protocol used for the simplex PCR reaction amplifying the poultry (chicken and turkey) 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	50	

Annex C: Results of the homogeneity measurements

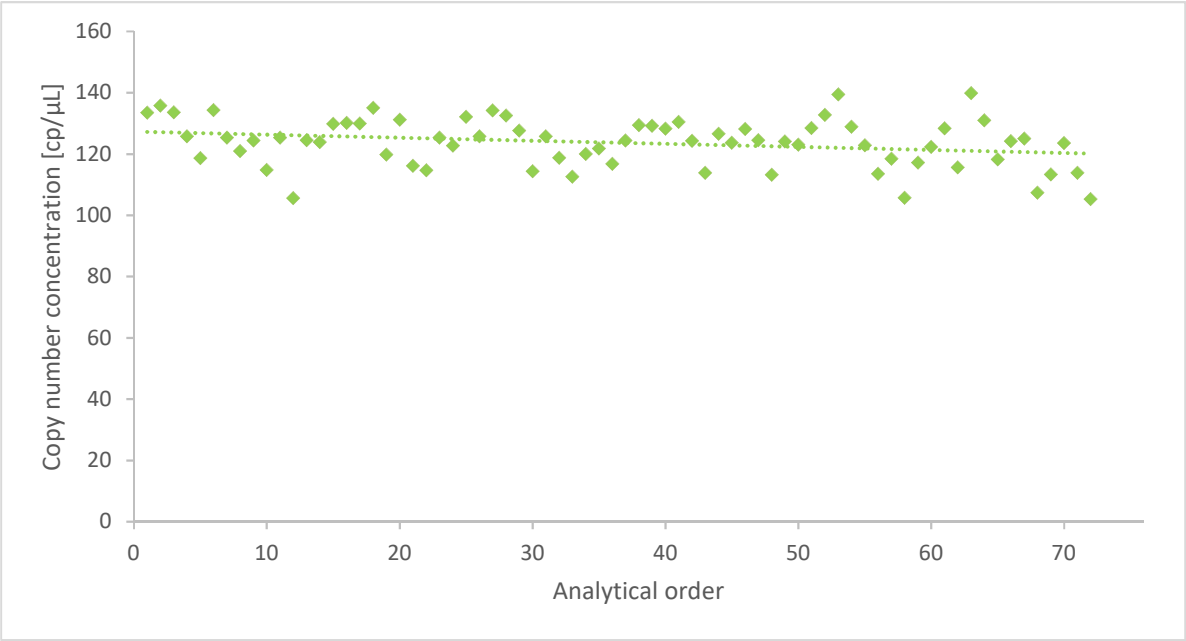

Figure C1: Individual copy number concentrations of ERM-AD484ka in analytical order before trend correction.

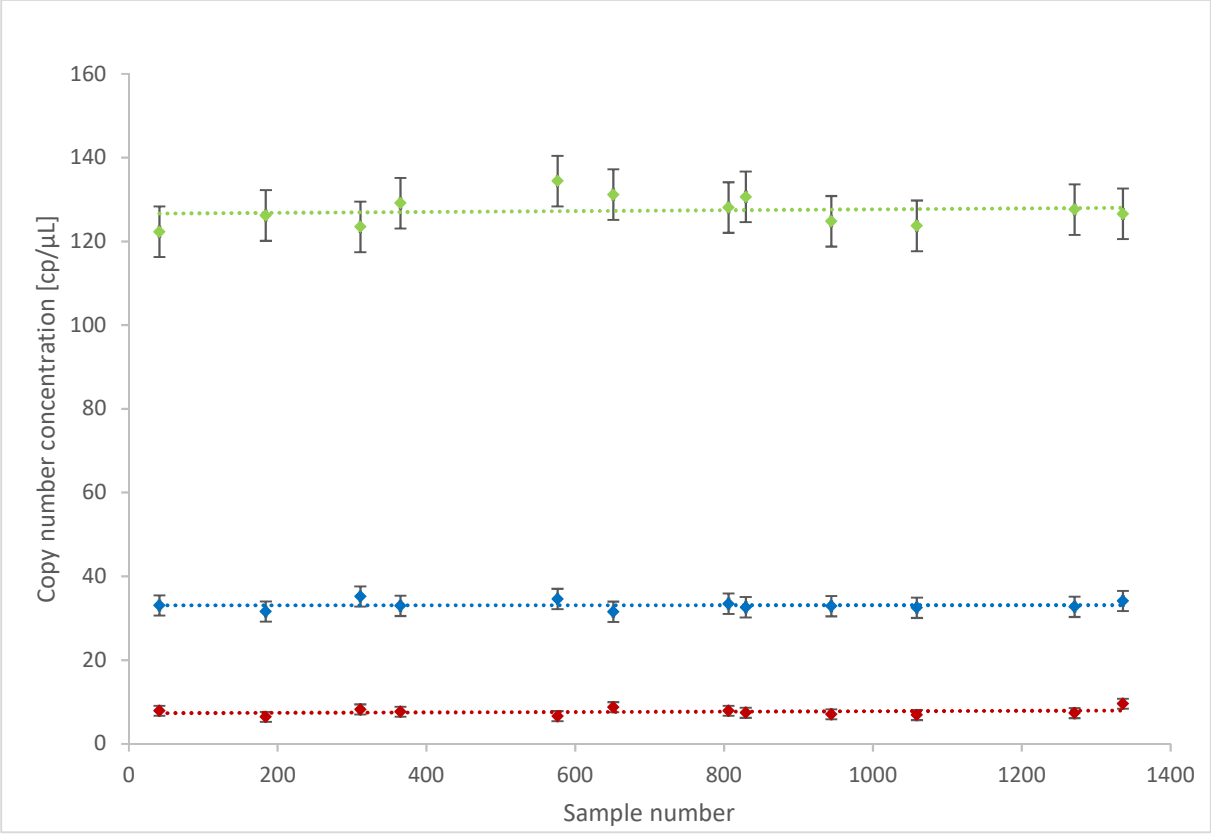

Figure C2: Data used for the assessment of the homogeneity. ERM-AD484ka after trend correction in green, ERM-AD484kb in blue and ERM-AD484kc in red. The error bars represent the 95 % confidence interval of the means of each unit based on the within-group standard deviation as obtained by one-way ANOVA.

Annex D: Results of the short-term stability measurements

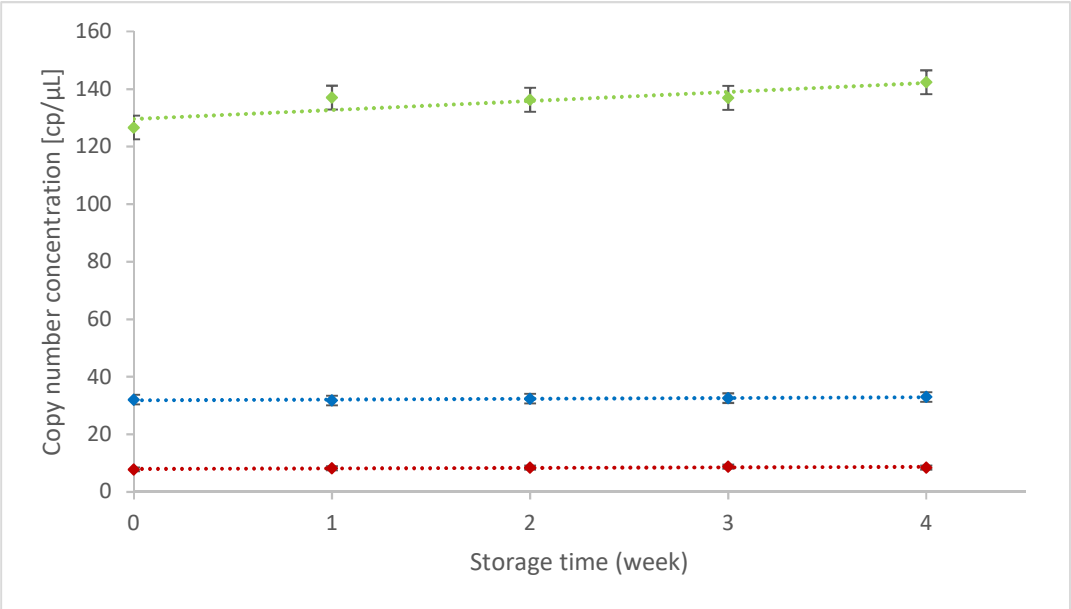

Figure D1: Data used for the assessment of the short-term stability at -20 °C. ERM-AD484ka in green, ERM-AD484kb in blue and ERM-AD484kc in red. The error bars represent the 95 % confidence interval of the means of each unit based on the within-group standard deviation as obtained by one-way ANOVA.

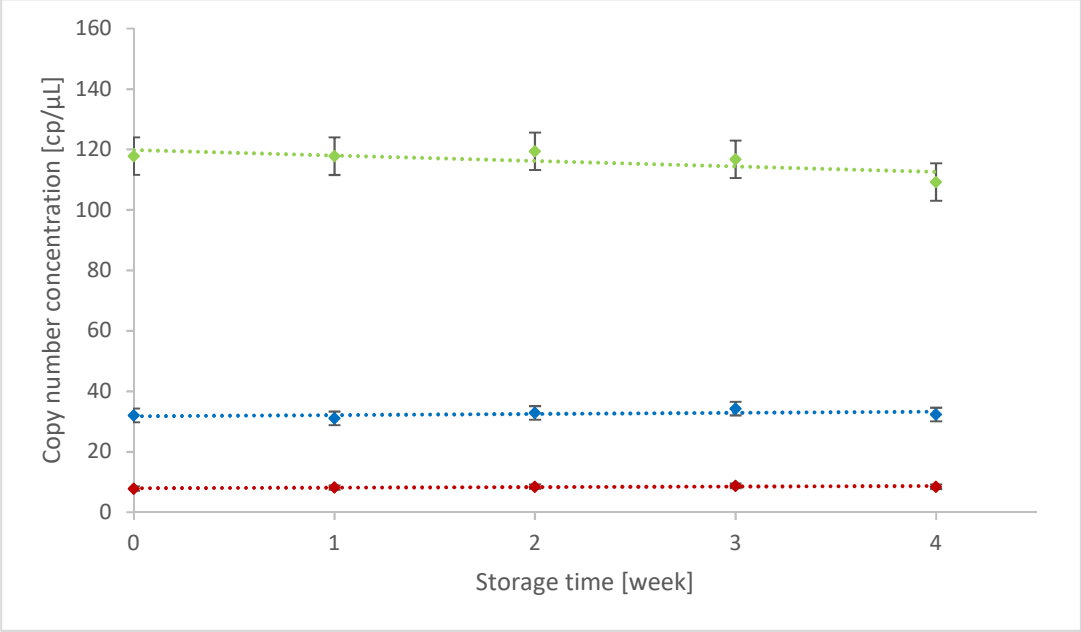

Figure D2: Data used for the assessment of the short-term stability at +4 °C. ERM-AD484ka in green, ERM-AD484kb in blue and ERM-AD484kc in red. The error bars represent the 95 % confidence interval of the means of each unit based on the within-group standard deviation as obtained by one-way ANOVA.

Annex E: Results of the long-term stability measurements

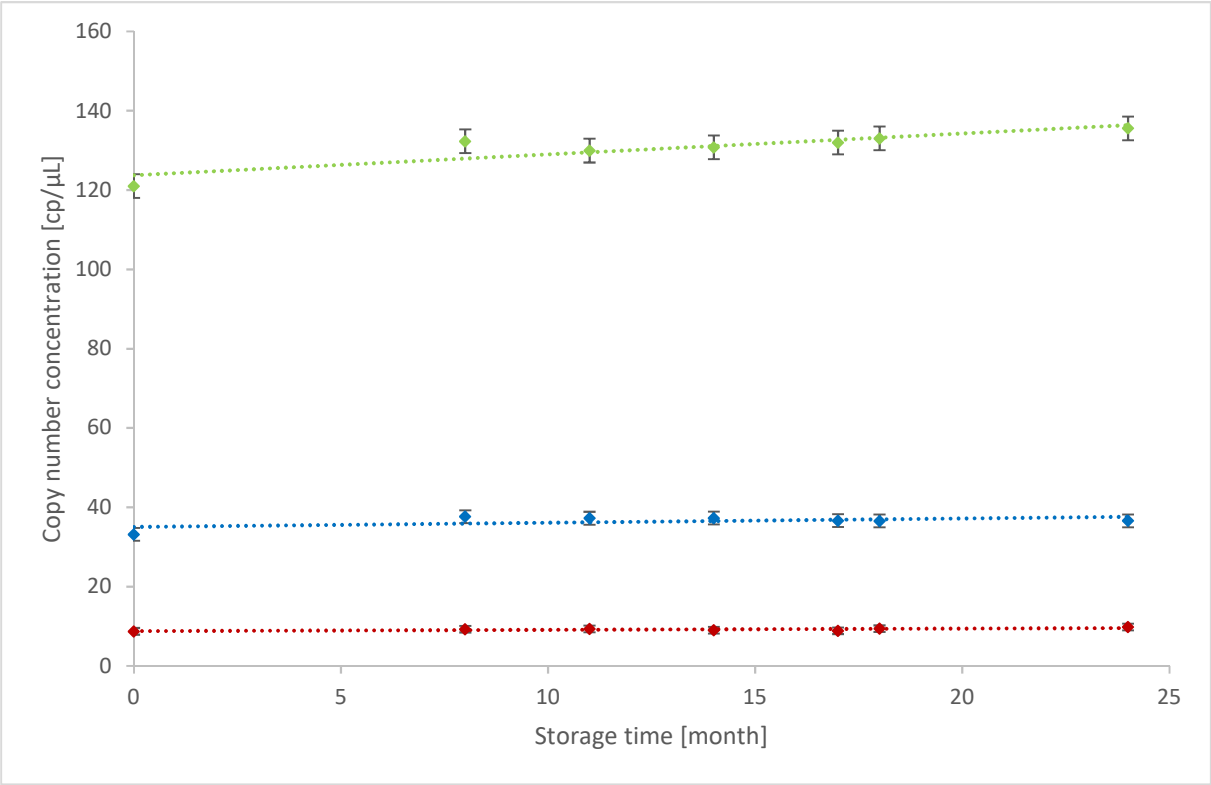

Figure E1: Data used for the assessment of the long-term stability at -20 °C. ERM-AD484ka in green, ERM-AD484kb in blue and ERM-AD484kc in red. The error bars represent the 95 % confidence interval of the means of each unit based on the within-group standard deviation as obtained by one-way ANOVA.

Annex F: Results of the repeated use measurements

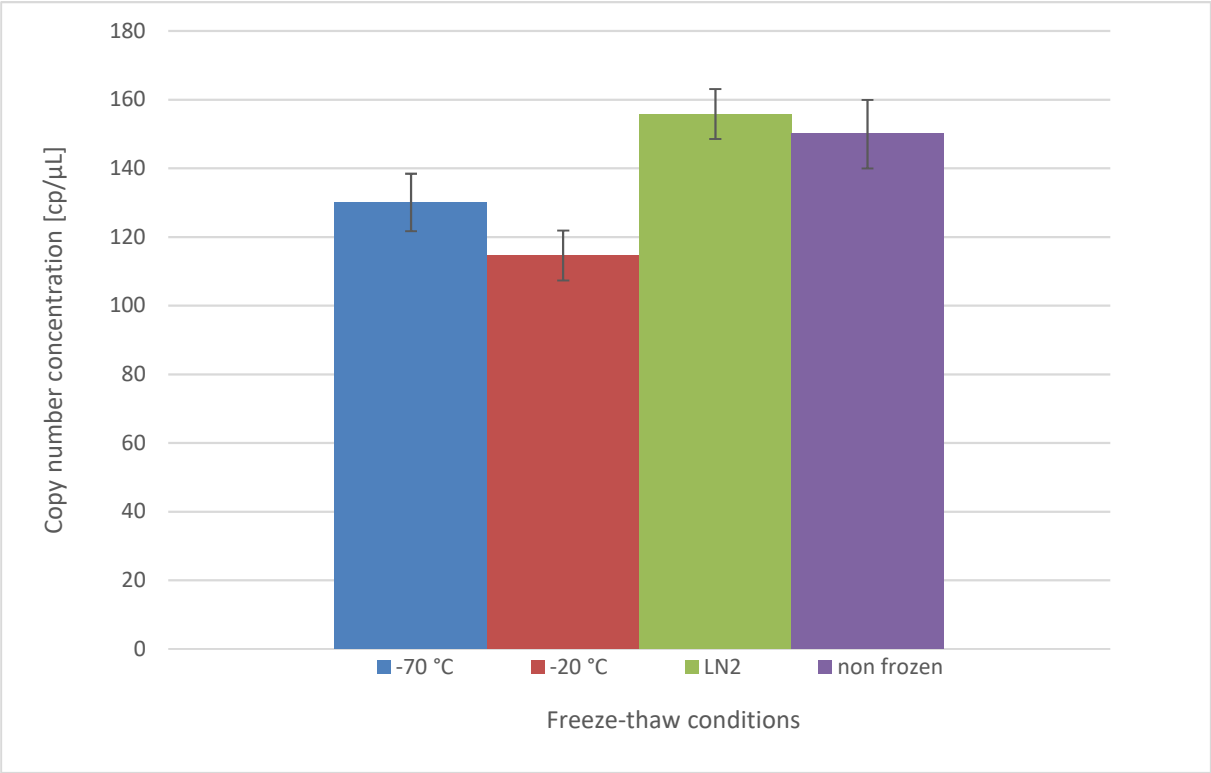

Figure F1: Data of the pilot batch with the same target concentration as ERM-AD484ka used for the assessment of the freeze-thaw study. Each bar represents the mean of four measurement results obtained by ddPCR. The error bars represent the standard deviation of the mean. Data for samples frozen at -20 °C in red, for samples frozen at -70 °C in blue, for samples frozen in LN in green and the non frozen material in purple.

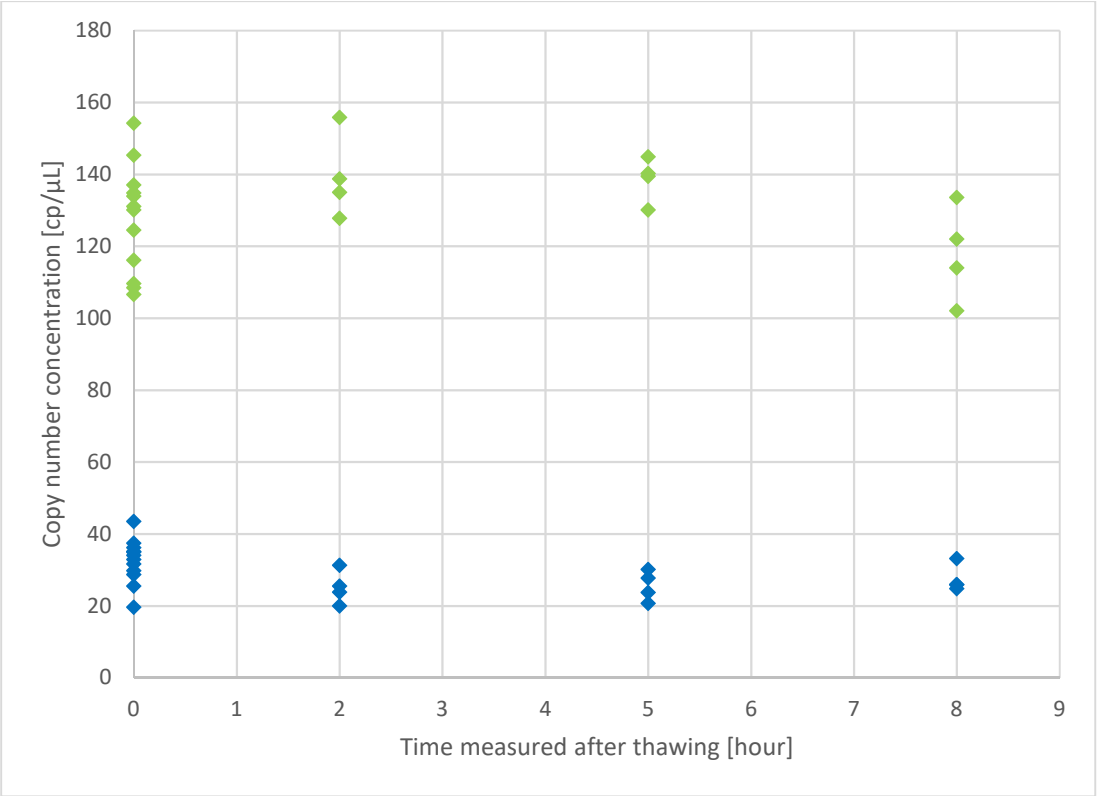

Figure F2: Data of ERM-AD484ka and ERM-AD484kb used for the assessment of the dedicated short-term stability. Each point represents a single measurement result obtained by cdPCR. ERM-AD484ka in green and ERM-AD484kb in blue.

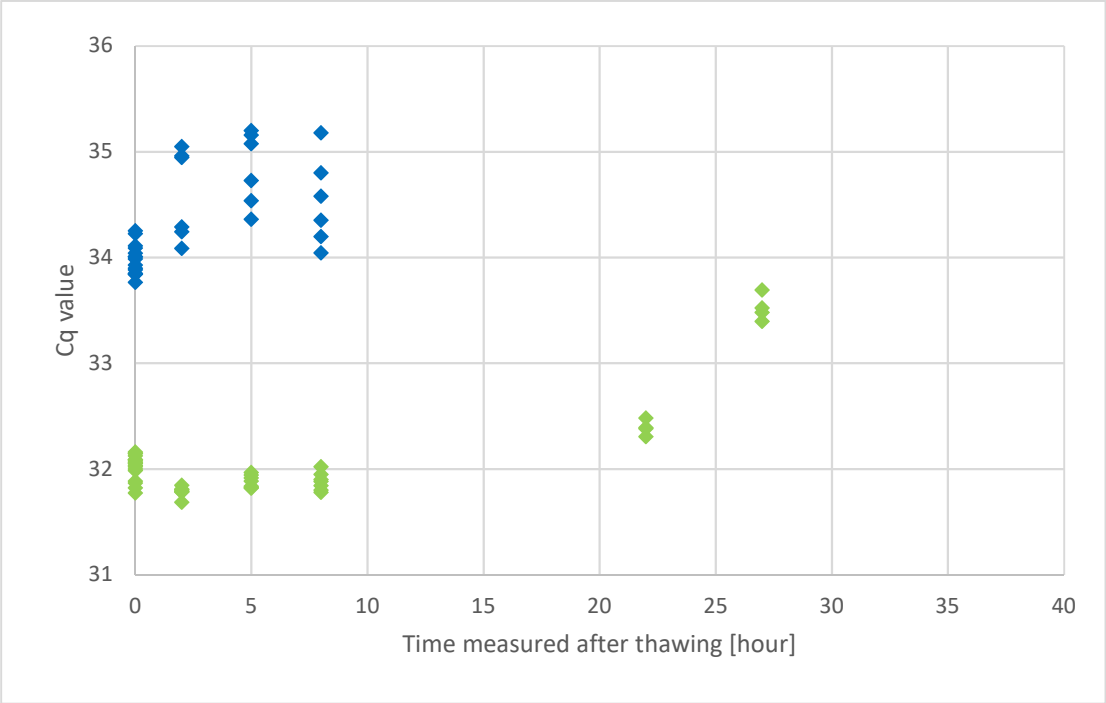

Figure F3: Data of ERM-AD484ka used for the assessment of the dedicated short-term stability. Each point represents a single measurement result obtained by qPCR. ERM-AD484ka in green and ERM-AD484kb in blue.

Annex G: Results of the characterisation measurements

Table G1: Individual results of the characterisation measurements of ERM-AD484ka

Laboratory code / Replicates [cp/µL]	L02-ddPCR	L03-ddPCR	L04-ddPCR	L05-ddPCR	L01-cdPCR	L05-cdPCR	*Results not used for value assignment*	L02-cdPCR	L03-cdPCR
replicate 1	125.75	133.37	129.79	125.14	138.44	93.65		32.59	82.59
replicate 2	110.44	132.52	126.01	118.82	122.36	110.46		21.90	75.23
replicate 3	140.57	130.86	134.11	131.63	123.60	98.12		25.75	67.34
replicate 4	111.31	128.19	131.81	126.86	128.54	100.35		34.19	72.18
replicate 5	108.73	131.49	119.84	122.41	131.67	101.79		28.34	84.36
replicate 6	122.96	120.58	131.58	133.22	111.60	106.85		21.04	77.75
replicate 7	108.96	132.38	127.01	122.38	117.50	110.68		17.45	81.26
replicate 8	108.90	127.27	121.57	130.94	140.22	121.16		22.13	74.63
replicate 9	133.48	149.51	131.41	133.04	123.23	136.18		23.79	70.66
replicate 10	134.71	128.20	141.05	120.26	123.65	118.03		20.51	69.33
replicate 11	127.77	127.05	130.51	127.36					
replicate 12	125.42	127.70	138.07	138.30					
replicate 13	121.80	131.39	143.77	117.35					
replicate 14	131.26	143.88	138.37	131.66					
replicate 15	120.76	135.58	124.24	115.38					
replicate 16	128.71	140.94	118.19	129.78					
mean [cp/µL]	122.60	132.56	130.46	126.53	126.08	109.73		24.77	75.53
Standard deviation [cp/µL]	10.30	7.14	7.48	6.59	8.86	12.71		5.43	5.84

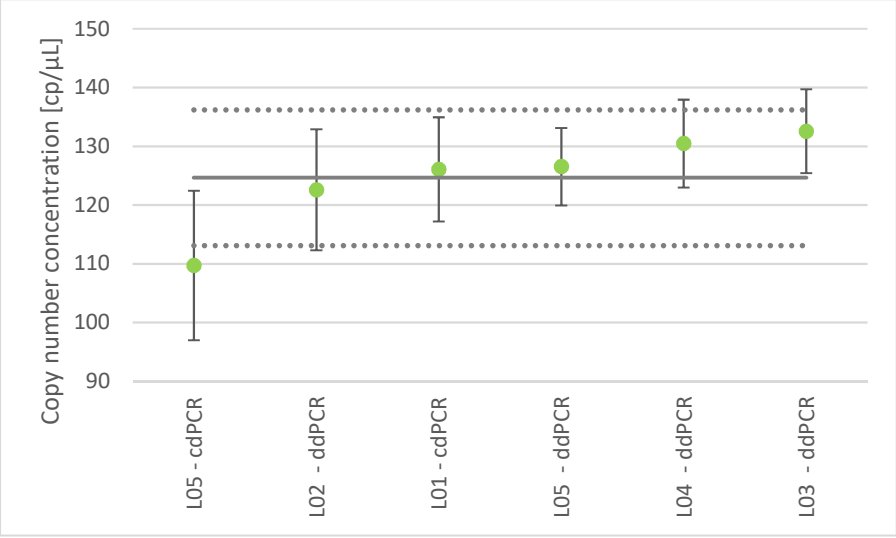

Figure G1: Results of the characterisation study of ERM-AD484ka. Continuous line: certified value; dashed line: expanded uncertainty with k = 2; error bars: standard deviation of each dataset.

Table G2: Individual results of the dPCR characterisation measurements of ERM-AD484kb

Laboratory code / Replicates [cp/µL]	L02-ddPCR	L03-ddPCR	L04-ddPCR	L05-ddPCR	L01-cdPCR	L05-cdPCR	*Results not used for value assignment*	L02-cdPCR	L03-cdPCR
replicate 1	29.68	34.38	35.51	30.58	24.84	37.54		3.29	7.34
replicate 2	37.45	32.52	31.98	34.08	31.86	35.48		5.36	1.17
replicate 3	26.25	36.06	37.30	28.36	29.35	38.87		6.13	7.99
replicate 4	32.80	29.53	34.65	32.19	26.53	48.27		5.04	6.22
replicate 5	31.54	31.30	33.87	33.44	30.28	36.66		7.87	5.00
replicate 6	27.58	32.75	32.66	33.66	31.39	46.16		5.07	6.93
replicate 7	33.07	36.17	33.08	30.62	33.64	35.44		3.29	4.76
replicate 8	27.52	32.98	32.42	31.85	40.40	39.05		3.90	2.53
replicate 9	32.04	31.38	29.20	30.86	25.07	36.22		5.08	15.76
replicate 10	31.16	33.93	27.59	37.06	26.25	33.45		4.43	7.78
replicate 11	34.83	31.24	27.50	28.43					
replicate 12	28.58	29.66	29.33	28.48					
replicate 13	29.34	30.42	28.72	27.68					
replicate 14	33.87	39.46	37.21	25.08					
replicate 15	32.84	35.82	32.97	29.30					
replicate 16	31.15	30.94	32.93	26.89					
Mean [cp/µL]	31.23	33.03	32.31	30.53	29.96	38.72		4.95	6.55
Standard deviation [cp/µL]	2.98	2.77	3.11	3.10	4.76	4.80		1.38	3.94

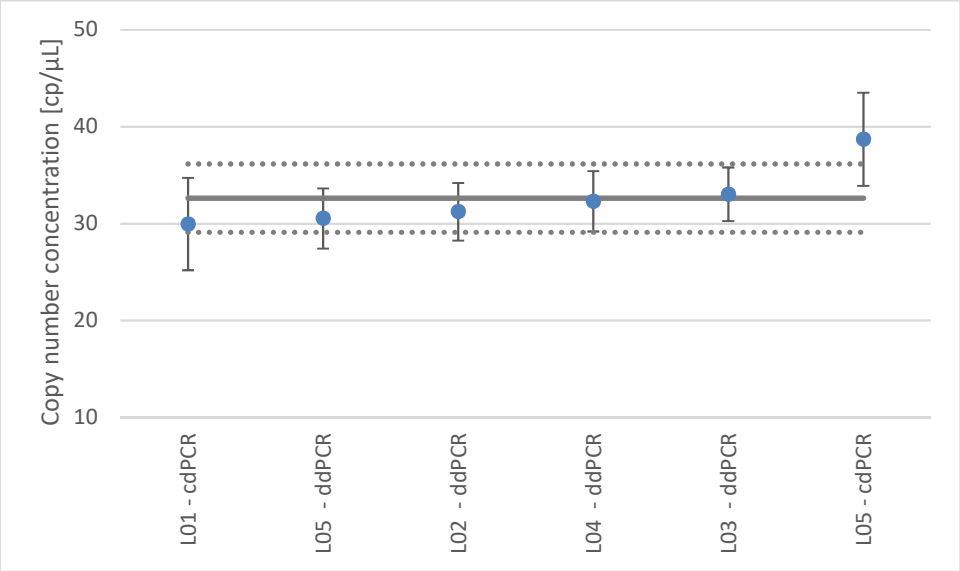

Figure G2: Results of the characterisation study of ERM-AD484kb. Continuous line: certified value; dashed line: expanded uncertainty with k = 2; error bars: standard deviation of each dataset.

Table G3: Individual results of the dPCR characterisation measurements of ERM-AD484kc

Laboratory code / Replicates [cp/µL]	L02-ddPCR	L03-ddPCR	L04-ddPCR	L05-ddPCR	L01-cdPCR	L03-cdPCR	L05-cdPCR	*Results not used for value assignment*	L02-cdPCR
replicate 1	7.71	8.28	9.00	7.95	6.04	8.94	9.14		1.14
replicate 2	7.07	7.07	7.00	8.19	11.47	6.20	10.34		1.58
replicate 3	6.14	8.13	8.09	7.96	6.60	6.69	11.32		2.87
replicate 4	7.42	8.35	9.89	7.74	7.21	7.91	11.06		2.19
replicate 5	9.27	8.53	8.28	8.17	4.46	5.15	9.04		0.57
replicate 6	7.13	6.39	9.26	7.30	3.72	4.38	4.34		2.24
replicate 7	8.07	11.51	7.72	7.91	11.37	6.77	8.53		1.75
replicate 8	6.63	10.17	7.11	6.74	8.26	8.04	6.71		1.15
replicate 9	6.98	9.09	8.03	5.21	6.94	6.71	7.58		0.56
replicate 10	6.89	7.35	7.97	5.71	4.80	2.81	10.31		3.27
replicate 11	/	9.07	8.72	11.22					
replicate 12	5.67	9.42	8.23	6.87					
replicate 13	6.01	9.25	10.99	6.14					
replicate 14	6.73	9.57	6.76	7.98					
replicate 15	6.33	6.77	7.42	6.07					
replicate 16	4.07	11.92	7.70	7.66					
Mean [cp/µL]	6.76	8.80	8.26	7.43	7.09	6.36	8.84		1.73
Standard deviation [cp/µL]	1.17	1.56	1.11	1.39	2.66	1.83	2.16		0.92

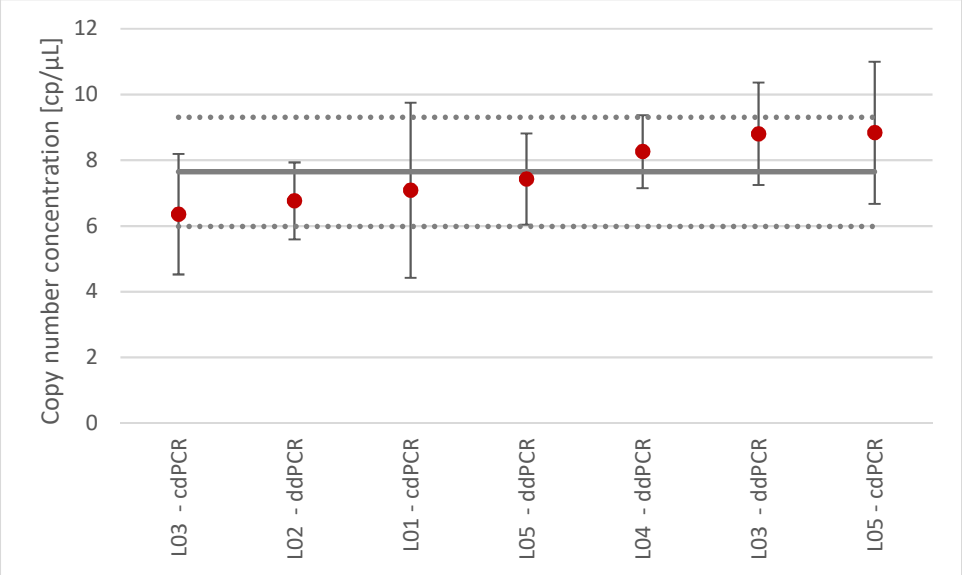

Figure G3: Results of the characterisation study of ERM-AD484kc. Continuous line: certified value; dashed line: expanded uncertainty with k = 2; error bars: standard deviation of each dataset.

European Commission

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