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CERTIFICATION REPORT

The certification of the mass of lambda DNA in a solution

Certified Reference Material: ERM®-AD442k

EUR 27160 EN - 2015

European Commission
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Institute for Reference Materials and Measurements (IRMM)

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Abstract
This report describes the processing and certification of genomic lambda deoxyribonucleic acid (DNA) in a solution. ERM®-AD442k is certified for its lambda DNA mass, expressed in ng/µL. The DNA copy number concentration in cp/µL is provided as an indicative value. The material was produced according to ISO Guide 34:2009.
A volume of approximately 400 mL of lambda DNA at an approximate concentration of 450 ng/µL was purchased from Promega Corporation and Benelux BV (Madison, USA and Leiden, NL). After homogenising and diluting this solution with TE buffer, 3100 vials of ERM-AD442k were produced. Each ERM-AD442k vial contains a certified DNA mass concentration of 57.53 ng/µL with an expanded combined uncertainty of 1.07 ng/µL. Using two different next generation sequencing (NGS) techniques (i.e. an Illumina platform and the GS Junior platform from Roche), the nucleic acid sequence of the lambda DNA in ERM-AD442k was verified. Non-lambda DNA sequences were identified in this material. The relative proportion of those sequences was estimated by NGS and further quantified by quantitative polymerase chain reaction (qPCR). Traces of the contaminating DNA, mainly coming from the Escherichia coli (E. coli) host used by Promega Corporation and Benelux BV to produce the lambda DNA, were negligible.
Between-vial homogeneity was quantified and stability during dispatch and storage were assessed in accordance with ISO Guide 35:2006 . Within-vial homogeneity was quantified to determine the minimum sample intake.
The certified DNA mass concentration value was obtained by ultraviolet (UV) spectrophotometry and the indicative DNA copy number concentration value by digital PCR (dPCR). The DNA copy number concentration measured by dPCR is consistent with the DNA mass concentration determined by UV spectrophotometry. The measurements were performed according to the scope of accreditation to ISO/IEC 17025:2005.
The material was characterised by an inter-laboratory comparison exercise performed by laboratories of demonstrated competence and with adherence to ISO/IEC 17025. Technically invalid results were removed; however no other outliers were eliminated on statistical grounds only.
Uncertainties of the certified and indicative values were calculated in accordance with the Guide to the Expression of Uncertainty in Measurement (GUM) [ ] and include uncertainties relating to possible inhomogeneity and instability, and to characterisation.
The material and its certified value are intended to be used for the calibration of DNA quantification methods, quality control and assessment of method performance. As any reference material, it can also be used to establish control charts or in validation studies. The indicative value of the material is, in contrast to the certified value, a value where the uncertainty was deemed too large to allow certification and is therefore less reliable than the certified value.
The CRM is available in Axygen maximum recovery polypropylene vial containing a nominal volume of 1.1 mL lambda DNA in solution. The minimum amount of sample to be used is 50 µL for UV spectrophotometry and 68 µL for dPCR. The CRM was accepted as European Reference Material (ERM®) after peer evaluation by the partners of the European Reference Materials consortium..

CERTIFICATION REPORT

The certification of the mass of lambda DNA in a solution

Certified Reference Material: ERM®-AD442k

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Summary

This report describes the processing and certification of genomic lambda deoxyribonucleic acid (DNA) in a solution. ERM®-AD442k is certified for its lambda DNA mass, expressed in ng/µL. The DNA copy number concentration in cp/µL is provided as an indicative value. The material was produced according to ISO Guide 34:2009 [1].

A volume of approximately 400 mL of lambda DNA at an approximate concentration of 450 ng/µL was purchased from Promega Corporation and Benelux BV (Madison, USA and Leiden, NL). After homogenising and diluting this solution with TE buffer, 3100 vials of ERM-AD442k were produced. Each ERM-AD442k vial contains a certified DNA mass concentration of 57.53 ng/µL with an expanded combined uncertainty of 1.07 ng/µL. Using two different next generation sequencing (NGS) techniques (i.e. an Illumina platform and the GS Junior platform from Roche), the nucleic acid sequence of the lambda DNA in ERM-AD442k was verified. Non-lambda DNA sequences were identified in this material. The relative proportion of those sequences was estimated by NGS and further quantified by quantitative polymerase chain reaction (qPCR). Traces of the contaminating DNA, mainly coming from the *Escherichia coli* (*E. coli*) host used by Promega Corporation and Benelux BV to produce the lambda DNA, were negligible.

Between-vial homogeneity was quantified and stability during dispatch and storage were assessed in accordance with ISO Guide 35:2006 [2]. Within-vial homogeneity was quantified to determine the minimum sample intake.

The certified DNA mass concentration value was obtained by ultraviolet (UV) spectrophotometry and the indicative DNA copy number concentration value by digital PCR (dPCR). The DNA copy number concentration measured by dPCR is consistent with the DNA mass concentration determined by UV spectrophotometry. The measurements were performed according to the scope of accreditation to ISO/IEC 17025:2005 [3].

The material was characterised by an inter-laboratory comparison exercise performed by laboratories of demonstrated competence and with adherence to ISO/IEC 17025. Technically invalid results were removed; however no other outliers were eliminated on statistical grounds only.

Uncertainties of the certified and indicative values were calculated in accordance with the Guide to the Expression of Uncertainty in Measurement (GUM) [4] and include uncertainties relating to possible inhomogeneity and instability, and to characterisation.

The material and its certified value are intended to be used for the calibration of DNA quantification methods, quality control and assessment of method performance. As any reference material, it can also be used to establish control charts or in validation studies. The indicative value of the material is, in contrast to the certified value, a value where the uncertainty was deemed too large to allow certification and is therefore less reliable than the certified value.

The CRM is available in Axygen maximum recovery polypropylene vial containing a nominal volume of 1.1 mL lambda DNA in solution. The minimum amount of sample to be used is 50 µL for UV spectrophotometry and 68 µL for dPCR.

The CRM was accepted as European Reference Material (ERM®) after peer evaluation by the partners of the European Reference Materials consortium.

The following values were assigned:

Lambda DNA in a solution		
	DNA mass concentration	
	Certified value [1] [ng/µL]	Uncertainty [2] [ng/µL]
Lambda DNA	57.5	1.1

1) As measured by UV spectrophotometry using an absorption coefficient for denatured DNA of 37 ng/µL (see Section 1.3 and Equation 1 in the Certification Report), including less than 0.02 ng/µL of non-lambda DNA (i.e. *E. coli* DNA). The certified value represents the unweighted mean value of the means of accepted sets of data, each set being obtained in a different laboratory. The certified value and its uncertainty are traceable to the International System of units (SI).

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

The DNA copy number concentration of the lambda DNA solution is also provided as an indicative value with a relative expanded combined uncertainty of 14.01 %.

Lambda DNA in a solution		
	Copy number concentration	
	Indicative value [1] [cp/µL]	Uncertainty [2] [cp/µL]
Lambda DNA	1.20×10^9	0.17×10^9

1) As measured by digital PCR using a set of primers and probe that amplifies a specific lambda DNA fragment and using the conditions described in Annex Table A1 – A3 of the Certification Report. The DNA copy number concentration was calculated using Equation 2 in the Certification Report. The indicative value is an unweighted mean value of the means of accepted sets of data, each set being obtained in a different laboratory. The indicative value and its uncertainty are traceable to the International System of units (SI).

2) The uncertainty of the indicative value is the expanded uncertainty with a coverage factor $k = 2.36$ corresponding to a level of confidence of about 95 % estimated in accordance with ISO/IEC Guide 98-3, Guide to the Expression of Uncertainty in Measurement (GUM:1995), ISO, 2008.

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Glossary

A	Adenine
A_{260}	Absorbance of DNA at 260 nm wavelength
A_{320}	Background absorbance at 320 nm wavelength
ANOVA	Analysis of variance
b	Slope in the equation of linear regression $y = a + bx$
BHQ1	Black Hole Quencher 1
BLAST	Basic Local Alignment Search Tool
bp	Base pair
c	Mass concentration $c = m / V$ (mass / volume)
C	Cytosine
Cl	Confidence level
Ct	Threshold of cycle
CRM	Certified reference material
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
ε	Absorption coefficient
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ERM®	Trademark of European Reference Materials
FAM	Fluorescein amidite
G	Guanine
GC	Guanine Cytosine
GUM	Guide to the Expression of Uncertainty in Measurements [4]
IRMM	Institute for Reference Materials and Measurements of the JRC
ISO	International Organization for Standardization
JRC	Joint Research Centre of the European Commission
k	Coverage factor
M	Molar mass
$MS_{between}$	Mean of squares between-vial from an ANOVA
MS_{within}	Mean of squares within-vial from an ANOVA
MW_{DNA}	Molecular weight of DNA
n.a.	Not applicable
NCBI	National Center for Biotechnology Information

NGS	Next generation sequencing
PCR	Polymerase chain reaction
dPCR	Digital PCR
qPCR	Quantitative PCR
rel	Index denoting relative figures (uncertainties etc.)
RNA	Ribonucleic acid
RSD	Relative standard deviation
r^2	Coefficient of determination of the linear regression
s	Standard deviation
s_{bb}	Between-vial standard deviation; an additional index "rel" is added when appropriate
$s_{between}$	Standard deviation between groups as obtained from ANOVA; an additional index "rel" is added as appropriate
SI	International System of Units
ssDNA	Single-stranded DNA
s_{within}	Standard deviation within groups as obtained from ANOVA; an additional index "rel" is added as appropriate
t	Time
T	Thymine
TaqMan®	*Thermus aquaticus* (*Taq*) DNA polymerase-based technology for fluorescent signal generation during real-time PCR
TE	Buffer containing TRIS and EDTA
TRIS	Tris(hydroxymethyl)aminomethane
u	Standard uncertainty
U	Expanded uncertainty
u^*_{bb}	Standard uncertainty related to a maximum between-vial 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-vial 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_{Δ}	Combined standard uncertainty of measurement result and certified value
u_{lts}	Standard uncertainty of the long-term stability; an additional index "rel" is added as appropriate
U_{meas}	Expanded measurement uncertainty

u_{sts}	Standard uncertainty of the short-term stability; an additional index "rel" is added as appropriate
UV	Ultraviolet
Δ_{meas}	Absolute difference between mean measured value and the certified value
$\nu_{MSwithin}$	Degrees of freedom of MS_{within}

1 Introduction

1.1 Background

Precise quantification of DNA is needed to optimise molecular techniques such as PCR, microarrays or NGS. Lambda DNA is frequently and widely used as a calibrant for measurements quantifying the mass of an extracted DNA in solution. Restricted lambda DNA fragments are also used as molecular markers for electrophoretic techniques such as gel or capillary electrophoresis. Popular DNA quantification kits, for example the Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies) and the DNAQF quantification kit (Sigma-Aldrich), rely on a calibration curve constructed with a lambda DNA standard to determine the DNA mass of an unknown sample. However, the DNA mass in such a standard is not a certified value and can vary from one lot to another. Consequently, potential bias in the DNA mass measurements could occur, leading to distorted results. Therefore, a reference material (RM) certified for its lambda DNA mass was needed.

For this purpose, the mass of the lambda DNA has been accurately determined by inter-laboratory comparison study, the lambda DNA mass variation from vial to vial and its stability investigated. Additionally the lambda DNA has been checked for its identity and purity and a certified value was established. The methods used to quantify the lambda DNA mass have different dynamic ranges. The UV spectrophotometric methods allow the quantification of DNA at a higher range (up to mg/µL), whereas dPCR methods can only quantify low concentration of DNA molecules (up to fg/µL) [5,6,7,8]. When applying dPCR methods, the sample had first to be highly diluted before being measured.

1.2 Choice of the material

Phage lambda, a virus that infects the bacterial species *E. coli*., is often chosen as a calibrant for DNA quantification measurements because it is a well-studied organism. Numerous scientific publications about *phage lambda*, its infection mechanism and its replication mechanism have been published [9,10,11,12,13]. The *phage lambda* particle is composed of a head, a tail and tail fibres. The genome of *phage lambda* consists of 1 chromosome, has a guanine-cytosine (GC) content of 49.9 % and 92 genes that encode 73 proteins. The lambda DNA chromosome is located in the head of the virus particle in its linear state, and has a double-stranded segment of 48490 bp and a single-stranded segment of 12 bp at the 5' site of both strands (called *cos* sites). When *phage lambda* infects its host *E. coli*, both *cos* sites of the chromosome anneal to each other and form a circular double-stranded phage DNA with a length of 48502 bp [12]. The reference sequence of *phage lambda* is established in the databank of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) of which the RefSeq number is NC_001416.1. Based on this reference sequence, primers and probe for dPCR assays can be designed [7].

The fact that *phage lambda* has a relatively high infection capability to *E. coli* and a high replication mechanism in *E. coli* [12,13,14], together with the easy and straightforward manipulation of *E. coli* in laboratories for different purposes (e.g. *in-vitro* mutagenesis, cloning process) [15,16,17,18], enables the production of large quantities of lambda DNA. Consequently, it was decided to use lambda DNA as material for ERM-AD422k. The lambda DNA used for production was purchased from Promega Corporation and Benelux BV (Madison, USA and Leiden, NL).

1.3 Design of the project

1.3.1 Methods

During the homogeneity, the stability and the characterisation studies, UV spectrophotometry and dPCR were used to determine the DNA mass concentration and the DNA copy number concentration (respectively). The vials for each study were selected using a random stratified sampling scheme which covered the whole batch.

1.3.1.1 UV spectrophotometry

UV spectrophotometry measures the intensity of light passing through a sample and compares it to the intensity of light of a blank solution. The intensity of this relative UV absorption will depend on the amount of macromolecules in the measured sample solution and is used as an indirect quantification method. The presence of RNA, proteins, single nucleotides, chemical and other components that also absorb UV light will bias the quantification of DNA. A mixture of denatured and undenatured DNA molecules in solution will also bias the DNA mass determination as dsDNA and ssDNA have different absorption coefficients (ε). To avoid the latter, dsDNA is completely denatured into ssDNA prior to the measurement by UV spectrophotometry. The DNA mass concentration is calculated using an absorption coefficient of 37 ng/µL [19] for ssDNA and based on Lambert-Beer law [20] (see Equation 1).

$$c_{DNA} = \varepsilon \times (A_{260} - A_{320}) \times D_{DNA} \qquad \textbf{Equation 1}$$

c_{DNA}	DNA mass concentration [ng/µL]
ε	Absorption coefficient ($\varepsilon = 37$ ng/µL for ssDNA)
A_{260}	Absorbance of DNA at 260 nm wavelength
A_{320}	Background absorbance at 320 nm wavelength
D_{DNA}	Dilution factor of the DNA mass concentration

In these studies, UV spectrophotometrical measurements were performed with denatured lambda DNA which was diluted 5 times gravimetrically with $T_1E_{0.01}$ buffer (1 mmol/L tris(hydroxymethyl)aminomethane [TRIS] and 0.01 mmol/L ethylenediamine tetraacetic acid [EDTA] at pH 8.0) and with NaOH that had a final concentration of 0.2 M. Absorbance at the wavelength of 230 nm (A_{230}), 260 nm (A_{260}), 280 nm (A_{280}) and 320 nm (A_{320}) were measured in a volume of 100 µL. Each vial was analysed in triplicate. The DNA purity was found acceptable if the A_{260}/A_{280} ratio was between 1.8 and 2.0 and if the A_{260}/A_{230} ratio was between 2.0 and 2.2.

1.3.1.2 Digital PCR

In the digital PCR array used for this study, the samples were distributed in several panels containing each a large number of partitions. A PCR was carried out in each individual partition. If a target DNA sequence is present in a partition, it will be eventually amplified and generate a fluorescent signal that is measured by a digital camera. The ratio between positive (H) and total number of partitions (C) was used to estimate the amount of target DNA sequence present in the original solution (T_c). Poisson approximation, using a binomial distribution, is applied for those partitions that contain more than one target DNA sequence

(Equation 2 [6]). To ensure an homogenous distribution of the target DNA over a panel, enzymatic digestion of the DNA prior to PCR was performed.

The copy number concentration (T_c) is calculated as follows:

$$T_C = \left(\frac{D_F}{V}\right) \times \frac{\left(\log\left(1-\frac{H}{C}\right)\right)}{\left(\log\left(1-\frac{1}{C}\right)\right)}$$ **Equation 2**

T_C	Copy number concentration [cp/µL]
D_F	Final dilution factor of the original undigested DNA sample
V	Volume of a panel
H	Number of partitions with a positive PCR signal
C	Number of total analysed partitions

The BioMark™ HD System and the BioMark™ 12.765 Digital Arrays (both from Fluidigm, South San Francisco, US) were used for analysing ERM-AD442k in the homogeneity, the stability and the characterisation studies. A 12.765 Digital Array is composed of 12 panels, of which each has 765 partitions. One partition has an estimated reaction volume of 6.01 nL. The total estimated volume per panel (V) is 4.597 µL.

The simplex dPCR assay for lambda DNA was derived from Bhat *et al.* [7]. The primers and the probe target a region of the lambda DNA that has a GC content of around 60 % [7]. The probe is labelled at the 5' end with 6-FAM (fluorescein amidite) and at the 3' end with the Black Hole Quencher 1 (BHQ1). Using the Basic Local Alignment Search Tool (BLAST) version 2.2.29+ from NCBI, the sequence of the primers and probe were analysed against the *phage lambda* reference sequence (NC_001416.1) and an *E. coli* database. The primers and probe had 100 % of nucleic acid sequence identity with the *phage lambda* reference sequence.

TaqMan® Universal PCR MasterMix (2x) (Applied BioSystems, Lennik, BE) and 20x GE sample loading reagent (Fluidigm) were used in the dPCR assay. Details of the primers and probe and the dPCR conditions are described in Annex Table A1 – A3.

Lambda DNA in each vial was digested with restriction enzyme *Taq(α)I* (New England Biolabs, Leiden, NL), followed by qualitative analysis using gel electrophoresis. The digested lambda DNA was gravimetrically diluted into 2 independent dilution series, each targeting a nominal final DNA copy number concentration of 447 cp/µL [6,7]. In total, there were 8 dilution steps (from the digestion step to the step of the reaction mix), resulting in a dilution factor of about 10 million. In each dilution series, digested lambda DNA was measured 5 times. The replicates were spread at random over different BioMark™ 12.765 digital arrays. The results were analysed using the Fluidigm Digital PCR Analysis software.

1.3.2 Studies

Several studies were performed including homogeneity, short-term stability, long-term stability and characterisation studies. The details of these studies are described in the Section 4, 5 and 6. Since the DNA could be fragmentised after freeze-thaw cycles, the consistency of the DNA mass concentration of ERM-AD442k was analysed by UV

spectrophotometry after up to 10 cycles of freezing and thawing (see Section 5.4). Sequence identification analysis and purity assessment were also performed (see Section 3.3.2).

2 Participants

2.1 Project management and evaluation

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

2.2 Raw material provider

Promega Corporation, Madison, USA

Promega Benelux BV, Leiden, NL

2.3 Processing, homogeneity and stability study

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

2.4 Characterisation

2.4.1 Sequence identity of lambda DNA and purity assessment

Baseclear BV, Leiden, NL

Eurofins Medigenomix GmbH, Ebersberg, DE
(measurements under the scope of ISO/IEC 17025 accreditation DAkkS, accreditation number D-PL-13372-01-00)

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

2.4.2 UV spectrophotometry measurement (DNA mass concentration)

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

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

LGC Limited, Science and Innovation, Molecular and Cell Biology, Teddington, UK

Livsmedelsverket, Science Department, Uppsala, SE

National Institute of Biology (NIB), Department of Biotechnology and Systems Biology, Ljubljana, SI

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

Solvias AG, Kaiseraugst, CH

2.4.3 Digital PCR measurements (DNA copy number concentration)

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

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

LGC Limited, Science and Innovation, Molecular and Cell Biology, Teddington, UK

National Institute of Biology (NIB), Department of Biotechnology and Systems Biology, Ljubljana, SI

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

3 Material processing and process control

3.1 Origin/Purity of the starting material

Lambda DNA (lot number 0000015651, part number D152X) was purchased from Promega Corporation and Benelux BV. For the lambda DNA starting material production, *E. coli* K-12 GM119 strain was infected with *cI*857 *Sam7 phage lambda*. When this *phage lambda* reached its lytic state in the host, *phage lambda* particles were isolated and unmethylated lambda DNA was extracted from this isolate via several purification steps. In the *cI*857 *Sam*7 lambda DNA strain, there are 4 known mutations that are annotated in the NCBI reference sequence NC_001416.1 and that are summarised in Table 1.

Table 1. Four known mutations in the *cI*857 *Sam*7 lambda DNA strain.

Gene	Position in the reference sequence	Reference nucleotide	Mutation
*ind*1	37589	C	T
*cI*857	37742	C	T
NinH	43082	G	A
*Sam*7	45352	G	A

During the production of the lambda DNA starting solution, Promega Corporation and Benelux BV performed quality control tests checking for the presence of proteins, chemical residues and nucleases. The results showed absence of these components in the lambda DNA starting solution. The unmethylated status of the lambda DNA was also confirmed by Promega Corporation and Benelux BV.

A volume of approximately 400 mL of buffered solution (10 mM Tris-HCl, 1 mM EDTA and 10 mM NaCl, pH of 7.5) containing lambda DNA at an estimated DNA mass concentration of 450 ng/µL was purchased from Promega Corporation and Benelux BV. The lambda DNA starting solution was shipped cooled and stored at 4 °C until processing.

The sequence identity and purity of the lambda DNA were analysed using NGS techniques, by two independent expert sequencing service companies. Two different NGS platforms were used: 1) Illumina and 2) GS Junior with Titanium series chemistry from Roche. The results of the NGS and qPCR analyses are given in Section 3.3.2.3. Nevertheless non-lambda (i.e. *E. coli*) DNA sequences were found in a very small amount and further quantification of these non-lambda DNA sequences was performed using qPCR. A negligible amount of *E. coli* DNA sequences was quantified.

3.2 Processing

DNA can aggregate and become viscous [21,22], therefore care needs to be taken to avoid inhomogeneous distribution of the DNA mass concentration between vials. To reduce the viscosity and the possibility of the aggregation of the lambda DNA, a nominal target DNA mass concentration of 50 ng/µL was chosen for the production of ERM-AD442k. A method for homogenising the lambda DNA solution was developed and assessed. Prior to the processing of ERM-AD442k, several tests were performed to determine the optimal conditions for the processing of the CRM, concerning e.g. the incubation temperature,

measures to avoid the formation of DNA aggregates and sedimentation. The optimal processing conditions, including the silanisation of glass bottles, were identified and applied in the processing of ERM-AD442k, as described in this Section. The processing scheme of ERM-AD442k is shown in Figure 1.

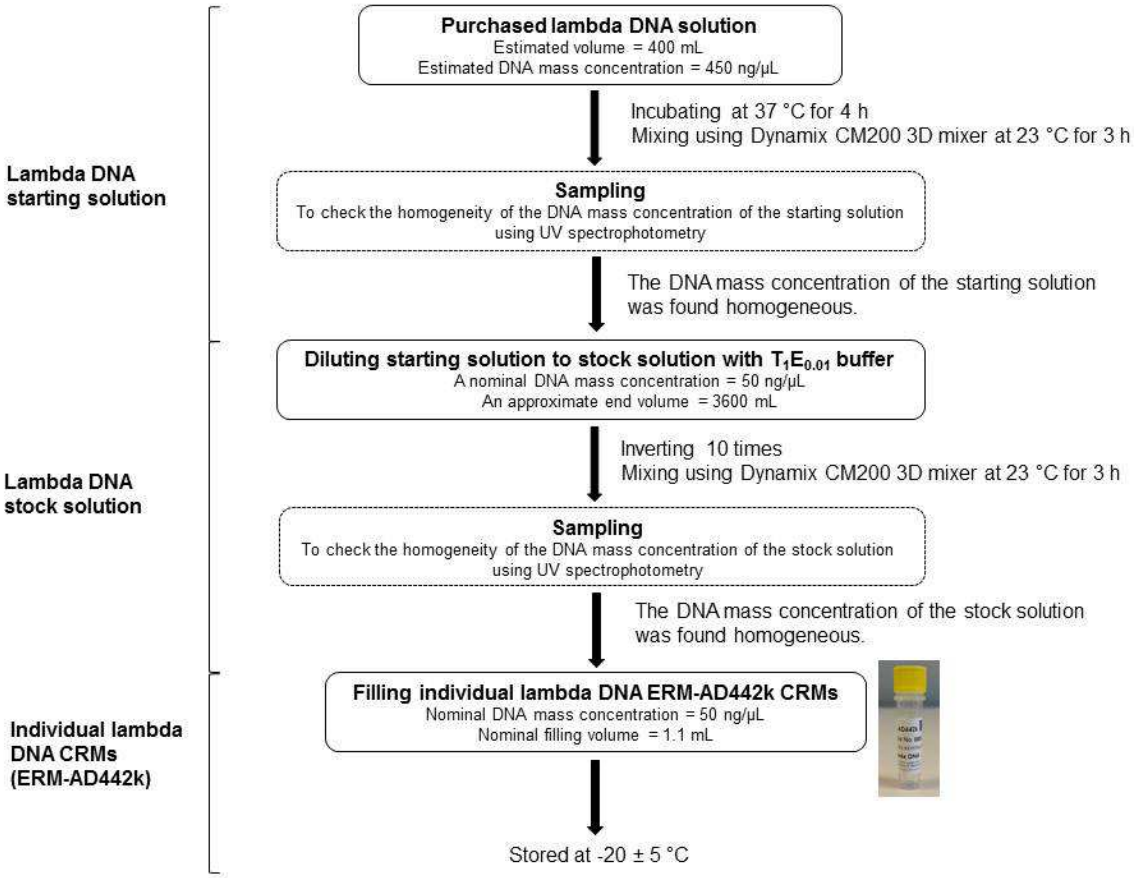

Figure 1. Processing scheme of ERM-AD442k.

3.2.1 Homogenising of the lambda DNA starting solution

The purchased lambda DNA starting solution was homogenised by incubating statically at 37 °C for 4 h, followed by a mixing step using Dynamix CM200 3D mixer (WAB, Basel, CH) at 23 °C for 3 h. At 3 depth levels of the solution (i.e. at the top, the middle and the bottom of the solution), a volume of approximately 50 µL was sampled to assess the DNA mass concentration of the solution with UV spectrophotometry using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, US). Absorbances at the 230 nm (A_{230}), 260 nm (A_{260}) and 280 nm (A_{280}) wavelength were measured. The DNA mass concentration was automatically calculated by the software of the NanoDrop 1000 spectrophotometer (Thermo Scientific), using an absorption coefficient of 50 ng/µL for dsDNA. The results are presented in Table 2. The average DNA mass concentration of all 3 depth levels of the lambda DNA starting solution was 450.8 ng/µL and its standard deviation was 3.7 ng/µL. The average ratio of A_{260}/A_{280} was 1.88 ± 0.01 and the average A_{260}/A_{230} ratio was 2.01 ± 0.01. Both ratios were in the acceptable DNA purity range. However, these values do not exclude traces of contaminating proteins or chemical residues from the host co-purified with the lambda DNA.

Table 2. Homogeneity test on the DNA mass concentration (c_{DNA}) of the lambda DNA starting solution after mixing. *s*: standard deviation

Intake location in the lambda DNA starting solution	Number of replicates	Mean c_{DNA} ± *s* [ng/µL]	Mean A_{260}/A_{280} ± *s* [*A*]	Mean A_{260}/A_{230} ± *s* [*A*]
top	3	449.1 ± 2.0	1.88 ± 0.01	2.01 ± 0.01
middle	3	454.5 ± 3.7	1.88 ± 0.01	2.01 ± 0.01
bottom	3	448.8 ± 2.6	1.88 ± 0.01	2.01 ± 0.01

The data were evaluated using a one-way analysis of variance (ANOVA). No significant difference between depth levels was observed at a confidence level of 99 %, indicating that the DNA mass concentration of the lambda DNA starting solution was homogenous. The degradation of the lambda DNA in the starting solution after mixing was assessed using gel electrophoresis with 1 % ethidium bromide-stained agarose gel. No DNA degradation was observed (data not shown).

3.2.2 Preparation of lambda DNA stock solution for filling

The lambda DNA starting solution was found to be homogeneous and after its DNA mass concentration was determined, a lambda DNA stock solution was prepared by diluting to a nominal DNA mass concentration of 50 ng/µL with sterilised $T_1E_{0.01}$ buffer. This results in a volume of approximately 3600 mL ready for the preparation of the CRMs. A silanised glass bottle was used for the lambda DNA stock preparation to minimise the DNA adsorption to glass. The bottle of the stock solution was inverted 10 times, before mixing by the Dynamix CM200 3D mixer at 23 °C for 3 h. At 4 depths of the stock solution (i.e. top, upper middle, lower middle and bottom), a 100 µL volume of lambda DNA stock solution was sampled for a homogeneity check using UV spectrophotometry, as above (Table 3). The average DNA mass concentration of all 4 depth levels of the stock solution was 59.4 ng/µL with a standard deviation of 0.8 ng/µL. The average ratio of A_{260}/A_{280} was 1.94 ± 0.04 and the average ratio of A_{260}/A_{230} was 2.16 ± 0.05. Both ratios were in the acceptable DNA purity range. Both ratios increased mildly in comparison with the ratios in Table 2, due to an increased proportion of $T_1E_{0.01}$ buffer to lambda DNA in the stock solution. The A_{260}/A_{280} ratio indicates that the amount of contaminants in the lambda DNA starting solution was higher than in the stock solution.

Table 3: Homogeneity test on the DNA mass concentration (c_{DNA}) of the lambda DNA stock solution after mixing. *s*: standard deviation

Intake location in the lambda DNA stock solution	Number of replicates	Mean c_{DNA} ± *s* [ng/µL]	Mean A_{260}/A_{280} ± *s* [*A*]	Mean A_{260}/A_{230} ± *s* [*A*]
top	3	58.7 ± 0.6	1.93 ± 0.03	2.19 ± 0.03
upper middle	3	59.2 ± 0.5	1.97 ± 0.04	2.12 ± 0.03
lower middle	3	60.4 ± 0.9	1.95 ± 0.06	2.24 ± 0.03
bottom	3	59.4 ± 0.3	1.91 ± 0.02	2.15 ± 0.03

The data was evaluated using a one-way ANOVA. No significant difference between depth levels was observed at a confidence level of 99 %, indicating the DNA mass concentration of the lambda DNA stock solution was homogeneous. The degradation of the lambda DNA in the stock solution after mixing was assessed using gel electrophoresis with 1 % ethidium bromide-stained agarose gel. No DNA degradation was observed (data not shown).

3.2.3 Preparation of individual lambda DNA CRMs

Once the homogeneity of the lambda DNA mass concentration in the stock solution was confirmed, 3100 sterile Axygen maximum recovery polypropylene vials (Corning, Tewksbury MA, US) were filled using semi-automated Opus® dispensers (Hirschmann Laborgeräte GmbH & Co. KG, Eberstadt, DE) under sterile conditions. The nominal filling volume was set to 1.1 mL per vial. After filling, all vials were tightly closed manually and checked visually to ensure correct filling.

Subsequently, vials for the homogeneity study, the stability studies, the characterisation study, the identification investigation, the purity assessment and the freeze-thaw investigation were selected according to a random stratified sampling scheme which covered the entire batch. The ERM-AD442k vials were stored at -20 ± 5 °C (i.e. the storage temperature).

3.3 Process control

During the processing of ERM-AD442k, the following aspects were investigated.

3.3.1 Production of individual lambda DNA CRMs

At IRMM the lambda DNA starting and stock solutions were mixed and analysed for their homogeneity concerning their DNA mass concentration using UV spectrophotometry. The DNA degradation was assessed using gel electrophoresis. This was followed by the filling of individual vials with lambda DNA in solution. The DNA mass concentration of both lambda DNA starting and stock solutions was homogeneous and the DNA in both solutions was not degraded prior to the filling of the vials (see Sections 3.2.1 and 3.2.2).

3.3.2 Sequence identification, molecular weight establishment and purity assessment

Two sequencing service companies used NGS techniques to determine the full nucleic acid sequence of the lambda DNA and to assess the purity of ERM-AD442k. Two different NGS techniques were used: Illumina NGS platform based on base-specific fluorescent sequencing, and GS Junior platform together with Titanium series chemistry (Roche) based on the pyrosequencing.

3.3.2.1 Sequence identification

The sequences obtained by both NGS techniques were compared to each other and to the *phage lambda* reference sequence in NCBI (NC_001416.1). Both NGS sequences were confirmed to be homologous to the lambda DNA sequence. The consensus sequence results in a lambda DNA sequence with a length of 48502 bp. A sequencing artefact was considered when a sequenced fragment did not align to the reference sequence and a variant in a sequence was considered as a true variant, when it occurred in both sequences and not in

the reference sequence (data not shown). Four variants were found to be true variants and are equal to the four previous known mutations listed in Table 1.

3.3.2.2 Molecular weight establishment

Based on the obtained assembled sequence from both NGS techniques, the molecular weight of the unmethylated lambda DNA in ERM-AD442k was established using Equation 3, resulting in a molecular weight of 29969028.65 g/mol.

$$MW_{DNA} = \{[(n_C \times M_{dCMP}) + (n_G \times M_{dGMP}) + (n_A \times M_{dAMP}) + (n_T \times M_{dTMP})] - [(n_{nt} - 1) \times M_{H_2O}]\} \times 2$$

Equation 3

n_C	Number of cytosine per single-stranded DNA
M_{dCMP}	Molar mass of deoxycytidine monophosphate (dCMP)
n_G	Number of guanine per single-stranded DNA
M_{dGMP}	Molar mass of deoxyguanosine monophosphate (dGMP)
n_A	Number of adenine per single-stranded DNA
M_{dAMP}	Molar mass of deoxyadenosine monophosphate (dAMP)
n_T	Number of thymine per single-stranded DNA
M_{dTMP}	Molar mass of deoxythymidine monophosphate (dTMP)
n_{nt}	Number of nucleotides in a single-stranded DNA
M_{H_2O}	Molar mass of water

The molar mass of nucleotide monophosphate (i.e. dCMP, dGMP, dAMP and dTMP) was calculated as diprotonated acid (i.e. as H_2PO_3). The molar mass of the nucleotide monophosphates and of water used in Equation 3 are displayed in Table 4. The accurate mass information was obtained from the National Institute of Standards and Technology (NIST) website: http://physics.nist.gov/PhysRefData/Compositions/index.html

(Date created: August 23, 2009 l Last updated: February 3, 2015)

Table 4. Molar mass of the nucleotide monophosphates and of water (used in Equation 3)

Nucleotide monophosphate/water	Chemical composition	Molar mass [g/mol]
dCMP	$C_9H_{14}N_3PO_7$	307.20
dGMP	$C_{10}H_{14}N_5PO_7$	347.22
dAMP	$C_{10}H_{14}N_5PO_6$	331.22
dTMP	$C_{10}H_{15}N_2PO_8$	322.21
Water	H_2O	18.02

The established molecular weight of the lambda DNA in ERM-AD442k enables the conversion from DNA copy number concentration (obtained from dPCR analysis) into a DNA mass concentration (see Equation 4) and vice versa.

$$c = \frac{T_C \times MW_{DNA} \times 10^9}{N_A}$$

Equation 4

c	DNA mass concentration [ng/µL]
T_C	Copy number concentration in the original undigested DNA sample [cp/µL]
MW_{DNA}	Molecular weight of DNA
N_A	Avogadro constant (6.02214129 x 10^{23} mol^{-1})

3.3.2.3 Purity assessment

The purity of ERM-AD442k was assessed using NGS techniques. All sequences were identified as being derived from either the lambda DNA or from contaminating DNA coming from the host. Because NGS is not a quantitative method, the amount of DNA sequences was roughly estimated based on the sequencing depth of coverage ratio for both DNA sequences. The proportion of non-lambda DNA (i.e. the host *E. coli* DNA) sequences to lambda DNA sequence was approximately 0.02 % (see Equation 5).

$$\iota_{NGS} = \left(\frac{n_{hostDNA}}{n_{lambdaDNA}} \right) \times \left(\frac{d_{hostDNA}}{d_{lambdaDNA}} \right) \qquad \textbf{Equation 5}$$

ι_{NGS}	Estimated impurity of host (i.e. *E. coli*) DNA by NGS [%]
$n_{hostDNA}$	Number of sequenced bp of the host DNA
$n_{lambdaDNA}$	Number of sequenced bp of the lambda DNA
$d_{hostDNA}$	Sequencing depth of coverage of the host DNA sequences
$d_{lambdaDNA}$	Sequencing depth of coverage of the lambda DNA sequence

To verify that these impurity traces were indeed derived from the host, two sets of primers and probes were designed for simplex qPCR assays based on the *E. coli* DNA sequences in the NGS data (named *E. coli* assay 1 and 2), using the software Primer Express software v.3.0.1 (Applied BioSystems). Additionally, two sets of primers and probe were designed to amplify a lambda DNA fragment in a simplex qPCR (named lambda DNA assay 1 and 2) [7]. The sequence details of the primer and probe to amplify lambda DNA and *E. coli* sequence fragments in the purity assessment are shown in Annex A.

Five ERM-AD442k vials were randomly selected from the whole batch for qPCR analysis, using ABI Prism 7900HT (Applied BioSystems). Each vial was analysed in triplicate using lambda DNA assay 1 and 2 and *E. coli* assay 1 and 2. The same amount of ERM-AD442k lambda DNA was used in each of the four qPCR assays. The differences in Ct values between the lambda DNA assays and the *E. coli* assays were averaged and the proportion of *E. coli* DNA trace sequences in the lambda DNA solution was calculated using Equation 6. A percentage of impurity of 0.13 % was calculated which is in the same range as the impurity percentage estimated by the relative sequencing depth of coverage of both DNA.

$$\iota_{qPCR} = \left(\frac{1}{2^{\overline{\Delta Ct}}} \right) \qquad \textbf{Equation 6}$$

ι_{qPCR}	Quantified impurity of host (i.e. *E. coli*) DNA by qPCR [%]
$\overline{\Delta Ct}$	Average of Ct value differences

Both techniques provide an indication of the impurity level in ERM-AD442k. Since the DNA copy number concentration of the lambda DNA based on dPCR is known, this percentage can be used to determine the DNA copy number concentration of the contaminating *E. coli* DNA sequences. Therefore the observed DNA copy number concentration of 1.57×10^6 cp/µL of the host DNA sequences can be further converted in DNA mass concentration. Moreover, knowing the size of the contaminating host DNA sequences (i.e. 12641 bp), a molecular weight of 7811108.86 g/mol was calculated for the host DNA sequences using Equation 3. A DNA mass concentration of 0.02 ng/µL was calculated for the host DNA sequences (see Equation 4).

4 Homogeneity

A key requirement for any certified reference material aliquoted into vials is the equivalence of the certified property between those vials. In this respect, it is relevant whether the variation between vials is significant compared to the uncertainty of the certified value. Consequently, ISO Guide 34 requires RM producers to quantify the between-vial variation. This aspect is covered in between-vial homogeneity studies.

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

4.1 Between-vial homogeneity

The between-vial homogeneity was evaluated to ensure that the certified and indicative values of the CRM are valid for all 3100 vials of the material, within the stated uncertainty.

The number of vials selected corresponds to approximately the cubic root of the total number of the vials produced and therefore 20 vials were selected using a random stratified sampling scheme covering the whole batch. For this, the batch was divided into 20 groups (each with a similar number of vials) and then one vial was selected randomly from each group. The content of each of these vials was measured 3 times by UV spectrophotometry under denatured conditions (as described in Section 1.3). They were also analysed by a simplex dPCR assay 10 times, 5 times per dilution series (according to Section 1.3). The measurements by UV spectrophotometry and dPCR were performed under repeatability conditions and in a randomised manner to be able to separate a potential analytical drift from a trend in the filling sequence.

4.1.1 Evaluation of trends in analytical and filling sequence

Regression analyses were performed to evaluate potential trends in the analytical sequence as well as trends in the filling sequence.

The correction of biases, even if they are statistically not significant, was found to combine the smallest uncertainty with the highest probability to cover the true value [23]. Correction of trends is therefore expected to improve the sensitivity of the subsequent statistical analysis through a reduction in analytical variation without masking potential between-vial heterogeneities. If the analytical sequence and the vial numbers are not correlated, trends significant on at least a 95 % confidence level can be corrected.

In the UV spectrophotometry and dPCR analyses, no trends in the filling sequence were observed. Whilst no trend in the analytical sequence was observed in the dPCR analyses, a trend in the analytical sequence was present in the UV spectrophotometry analyses (95 % confidence level). After removing one technical outlier (due to an air bubble), the analytical trend remained. The analytical trend was corrected according to Equation 7:

$$x_c = x_m - b \cdot i \qquad \textbf{Equation 7}$$

x_c = corrected result

x_m = measured result

b = slope of the linear regression

i = position of the result in the analytical sequence

4.1.2 Statistical evaluation of datasets

The trend-corrected UV spectrophotometry dataset and the dPCR dataset were assessed for consistency using Grubbs outlier tests at a confidence level of 99 % on the individual results and the unit means. No outlying individual result and no outlying unit mean in the dataset of UV spectrophotometry and of dPCR were detected. The results of both techniques are shown in Annex B.

Quantification of between-vial inhomogeneity for the UV spectrophotometry was undertaken by analysis of variance (ANOVA), which can separate the between-vial variation (s_{bb}) from the within-vial variation (s_{wb}). The latter is equivalent to the method repeatability if the individual samples are representative for the whole vial. In dPCR analysis, because of the two independent dilution series, 2-way ANOVA with replication was performed.

Evaluation by ANOVA requires vial means which follow at least a unimodal distribution and results for each vial that follow unimodal distributions with approximately the same standard deviations. Distribution of the vial means was assessed using histograms and normal probability plots. Minor deviations from unimodality of the individual values do not significantly affect the estimate of between-vial standard deviations.

It should be noted that $s_{bb,rel}$ and $s_{wb,rel}$ are estimates of the true standard deviations and are subject to random fluctuation. Therefore, the mean square between groups ($MS_{between}$) can be smaller than the mean square within groups (MS_{within}), resulting in negative arguments under the square root used for the estimation of the between-vial variation, whereas the true variation cannot be lower 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.* [24]. u^*_{bb} is comparable to the limit of detection of an analytical method, yielding the maximum inhomogeneity that might be undetected by the given study setup.

Method repeatability ($s_{wb,rel}$), between–vial standard deviation ($s_{bb,rel}$) and $u^*_{bb,rel}$ for UV spectrophotometry were calculated as:

$$s_{wb,rel} = \frac{\sqrt{MS_{within}}}{\bar{y}} \qquad \textbf{Equation 8}$$

$$s_{bb,rel} = \frac{\sqrt{\frac{MS_{between} - MS_{within}}{n}}}{\bar{y}} \qquad \textbf{Equation 9}$$

$$u^*_{bb,rel} = \frac{\sqrt{\frac{MS_{within}}{n}\sqrt[4]{\frac{2}{v_{MSwithin}}}}}{\bar{y}} \qquad \textbf{Equation 10}$$

MS_{within} mean square within a vial from an ANOVA

$MS_{between}$ mean square between-vial from an ANOVA

$\bar{y}$ mean of all results of the homogeneity study

n mean number of replicates per vial

$v_{MSwithin}$ degrees of freedom of MS_{within}

Method repeatability ($s_{wb,rel}$) and between–vial standard deviation ($s_{bb,rel}$) for dPCR were calculated as:

$$s_{wb,rel} = \frac{\sqrt{MS_{interaction} + MS_{within}}}{\bar{y}} \quad \textbf{Equation 11}$$

$$s_{bb,rel} = \frac{\sqrt{\frac{MS_{between} - MS_{interaction} - MS_{within}}{N}}}{\bar{y}} \quad \textbf{Equation 12}$$

$MS_{between}$ mean square between-vial from an ANOVA

$MS_{interaction}$ mean square interaction from an ANOVA

MS_{within} mean square within a vial from an ANOVA

N number of vials

$\bar{y}$ mean of all results of the homogeneity study

The results of the evaluation of the between-vial variation are summarised in Table 5.

Table 5: Results of the homogeneity study

Method	$s_{wb,rel}$ [%]	$s_{bb,rel}$ [%]	$u^*_{bb,rel}$ [%]	$u_{bb,rel}$ [%]
UV spectrophotometry	0.99	0.58	0.27	0.58
dPCR	11.42	1.75	1.18	1.75

The homogeneity study showed no outlying vial means or trends in the filling sequence for the UV spectrophotometry and the dPCR methods. Therefore, the between-vial standard deviation could be used as an estimate of u_{bb} for UV spectrophotometry as well as for dPCR. 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-vial homogeneity and minimum sample intake

The minimum sample intake is the minimum amount of sample that is representative for the whole vial and thus can be used in an analysis. The within-vial homogeneity is correlated to the minimum sample intake and therefore it follows that individual aliquots of a material, below the minimum sample intake will not contain the same amount of analyte. Sample sizes

equal or above the minimum sample intake guarantee the certified and the indicative value within its stated uncertainty. The minimum sample intake can be obtained from a dedicated study investigating a series of sample intakes or from the homogeneity/stability studies using a specific sample intake.

4.2.1 UV spectrophotometry

A dedicated minimum sample intake study was performed for UV spectrophotometry. To estimate the minimum sample intake, a series of measurements with decreasing amounts of sample for one randomly selected vial were performed. The following sample intakes were tested: 5 µL, 10 µL, 15 µL, 20 µL, 50 µL and 100 µL. For each sample intake, 3 subsamples of the vial were measured by UV spectrophotometry under repeatability conditions, and in a randomised manner. Each subsample was measured in triplicate. The measurement method was robust over the whole range of the sample intake tested and its repeatability was in the same range or better than the repeatability achieved during the material characterisation (Section 6). One technical outlier in the data of the minimum sample intake of 50 µL was observed due to an air bubble and excluded in the data sets before the data evaluation.

The obtained data sets (all sample intakes taken together) were first evaluated whether they follow a normal, or at least unimodal distribution. This was done by normal probability plots and histograms (if the data do not follow at least a unimodal distribution, the calculation of standard deviations is doubtful or impossible). All results were normally and unimodally distributed.

Furthermore, the results (all sample intakes taken together) were scrutinised for outliers using the single Grubbs-test. No outlier was detected. The minimum sample intake was established by comparison of variances obtained for 5 µL, 10 µL, 15 µL, 20 µL and 50 µL sample intakes with the variance obtained for 100 µL sample intake (F-test, 8 degrees of freedom, CI 95 %).

The obtained results are presented in Annex C, indicating that the minimum sample intake representative of ERM-AD442k using UV spectrophotometry is 50 µL. The sample intake gives an acceptable repeatability, demonstrating that the within-vial inhomogeneity no longer contributes to analytical variation.

4.2.2 Digital PCR

Homogeneity/stability experiments were performed using a 68 µL sample intake for dPCR. The sample intake gives an acceptable repeatability, demonstrating that the within-vial inhomogeneity no longer contributes to analytical variation.

In conclusion, the minimum amount of sample to be used is 50 µL for UV spectrophotometry and 68 µL for dPCR.

5 Stability

Time, temperature and radiation were regarded as the most relevant influences on the stability of the material. In addition, materials are stored and dispatched in the dark, thus eliminating the possibility of degradation by UV radiation. Therefore, only 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 material to the customers (short-term stability).

The stability studies were performed using an isochronous design [25]. In this approach, vials were stored for a specified length of time at different temperatures whereupon the vials were then moved to conditions where further degradation was assumed to be negligible (reference conditions). At the end of the isochronous storage, the vials were analysed simultaneously under repeatability conditions. Analysis of the material (after various exposure times and temperatures) under repeatability conditions greatly improves the sensitivity of the stability tests.

5.1 Short-term stability study

During the short-term stability study, vials were stored at -20 °C and 4 °C for 0, 1, 2 and 4 weeks. The reference temperature was set to -70 °C. Five vials per storage time/temperature combination were selected using a random stratified sampling scheme. At the end of the stability study each vial was analysed 3 times by UV spectrophotometry and 10 times by dPCR (as described as in Section 1.3). The measurements were performed under repeatability conditions, and in a randomised sequence to be able to separate a potential analytical drift from a trend over storage time.

The data were evaluated for each temperature individually. The results were screened for outliers using the single and double Grubbs test. Only one outlying individual result was found for the UV spectrophotometry at the temperature of 4 °C at 1 week. As no technical reason for the outlier could be found, all UV spectrophotometrical data were retained for statistical analysis and for the estimation of u_{STS}.

Furthermore, the data were evaluated against storage time and regression lines of DNA mass concentration or DNA copy number concentration versus time were calculated. The slopes of the regression lines were tested for statistical significance (loss/increase due to shipping conditions). For the DNA mass concentration and the DNA copy number concentration, the slopes of the regression lines were not significantly different from zero (at a 99 % confidence level) at both -20 °C and 4 °C. The material was therefore shown to be stable for 4 weeks at -20 °C and 4 °C. The results of the measurements are shown in Annex D.

It was concluded to ship the material frozen on dry ice to avoid thawing of the material during transport.

5.2 Long-term stability study

During the long-term stability study, vials were stored at -20 °C for 0, 4, 8, 12 and 18 months. The reference temperature was set to -70 °C. Five vials per storage time were selected using a random stratified sampling scheme. At the end of the stability study each vial was analysed 3 times by UV spectrophotometry and 10 times by dPCR (as described as in Section 1.3). The measurements were performed under repeatability conditions, in a random sequence to be able to separate any potential analytical drift from a trend over storage time.

The results were screened for outliers using the single and double Grubbs test. Only one outlying individual result was found for the UV spectrophotometry. As no technical reason for the outliers could be found, all UV spectrophotometrical data were retained for statistical analysis and for the estimation of u_{LTS}.

Furthermore, the data were plotted against storage time and linear regression lines of DNA mass concentration or DNA copy number concentration versus time were calculated. The slope of the regression lines was tested for statistical significance (loss/increase due to storage conditions). For the DNA mass concentration and the DNA copy number concentration, the slopes of the regression lines were not significantly different from zero (at a 99 % confidence level) for -20 °C. The material should therefore be stored at -20 °C. The results of the long term stability measurements are shown in Annex E.

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 report that there was no detectable degradation during the timeframe studied.

Uncertainties of stability during dispatch and storage were estimated as described in [26]. 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} are calculated as the product of the chosen transport time/shelf life and the uncertainty of the regression lines as:

$$u_{sts,rel} = \frac{RSD}{\sqrt{\sum \left(t_i - \bar{t}\right)^2}} \cdot t_{tt} \qquad \textbf{Equation 13}$$

$$u_{lts,rel} = \frac{RSD}{\sqrt{\sum \left(t_i - \bar{t}\right)^2}} \cdot t_{sl} \qquad \textbf{Equation 14}$$

RSD relative standard deviation of all results of the stability study

t_i time elapse at time point *i*

$\bar{t}$ mean of all t_i

t_{tt} chosen transport time (1 week at -20 ºC)

t_{sl} chosen shelf life (18 months at -20 ºC)

The following uncertainties were estimated:

- $u_{sts,rel}$, the uncertainty of degradation during dispatch. This was estimated from the -20 °C study. The uncertainty describes the possible change during a dispatch at -20 °C lasting one week.
- $u_{lts,rel}$, the stability during storage. This uncertainty contribution was estimated from the -20 °C study. The uncertainty contribution describes the possible degradation during 18 months storage at -20 °C.

The results of these evaluations are summarised in Table 6.

Table 6: Uncertainties related to stability during dispatch ($u_{sts,rel}$ calculated for a dispatch at -20 °C for 1 week) and storage ($u_{lts,rel}$ calculated for storage at -20 °C for 18 months).

ERM-AD442k	$u_{sts,rel}$ [%]	$u_{lts,rel}$ [%]
UV spectrophotometry	0.12	0.23
dPCR	0.60	1.99

After the certification study, the material will be included in IRMM's regular stability monitoring programme to determine their future stability, once a year.

5.4 Stability after freeze-thaw cycles

Since DNA can degrade after freeze-thaw cycles, the DNA intactness and the consistency of the DNA mass concentration of ERM-AD442k were studied. The approach of this study was similar to the isochronous design. Two vials were submitted to a certain number of freeze-thaw cycles (i.e. 0, 1, 3, 5, 7 and 10) and after each thawing session the vials were moved to the storage temperature (i.e. -20 °C). In total, 10 vials were selected based on a random stratified sampling scheme. The DNA intactness of each vial was analysed using gel electrophoresis. The DNA in none of the samples was found to be degraded (data not shown).

In order to check the consistency of the DNA mass concentration over 10 freeze-thaw cycles, the DNA of each vial was measured 3 times by UV spectrophotometry under denatured condition (as described as in Section 1.3). The measurements were performed under repeatability conditions, in a random sequence to be able to separate any potential analytical drift from a trend over number of freeze-thaw cycles. The obtained data were screened for outliers using the single and double Grubbs test. No outlying individual results were found. The data were evaluated against number of freeze-thaw cycles and regression lines of DNA mass concentration versus number of freeze-thaw cycles were calculated. The slopes of the regression lines were tested for statistical significance (loss/increase due to freeze-thaw conditions). For the DNA mass concentration, the slopes of the regression lines were significantly different from zero (99 % confidence level) at 10 times of freezing and thawing, but not significantly different from zero (99 % confidence level) at 5 and 7 times of freezing and thawing. The results are given in Annex F.

Therefore, it is recommended not to freeze-thaw ERM-AD442k more than 5 times in order to have consistency of the DNA mass concentration. Aliquoting ERM-AD442k is highly recommended after the first thawing and homogenisation.

6 Characterisation

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

The material characterisation was based on two inter-laboratory comparisons of expert laboratories that used two methods (UV spectrophotometry and/or dPCR). The DNA mass concentration of ERM-AD442k was measured by UV spectrophotometry. The measurements for DNA copy number concentration of ERM-AD442k was measured by dPCR using the BioMark™ HD System and the BioMark™ 12.765 Digital Arrays (both from Fluidigm). The inter-laboratory comparison approach allowed to negate laboratory bias, reducing the combined uncertainty.

6.1 Selection of participants

Five and seven laboratories were selected based on criteria that comprised both technical competence and quality management for UV spectrophotometry or dPCR measurements. Each laboratory was required to operate a quality system and to deliver documented evidence of its proficiency in the field of UV spectrophotometry and/or dPCR measurements. Having a formal accreditation was not mandatory, but meeting the requirements of ISO/IEC 17025 was obligatory.

6.2 Study setup

6.2.1 Measurements using UV spectrophotometry

Each laboratory received 6 vials of ERM-AD442k and was required to provide 36 results, 6 per vial. The units for material characterisation were selected using a random stratified sampling scheme which covered the whole batch. The sample preparations and measurements had to be spread over at least two days to ensure intermediate precision conditions. The mean value of the 6 replicates of a vial was considered as one independent measurement result.

6.2.2 Measurements using dPCR

Each laboratory received 6 vials of ERM-AD442k and was required to provide 60 results, 10 per vial. The vials for material characterisation were selected using a random stratified sampling scheme which covered the whole batch. The sample preparations and measurements had to be spread over at least two days and over different arrays to ensure intermediate precision conditions.

All laboratories used the BioMark™ HD System and the BioMark™ 12.765 Digital Arrays (both from Fluidigm). The lambda DNA of each vial was first digested, followed by the preparation of two independently gravimetrical dilution series. The final DNA concentration solution of each dilution series was analysed 5 times in a simplex dPCR. These 5 replicates were spread at random over different arrays (as described as in Section 1.3). The mean value of the 10 replicates of a vial was considered as one independent measurement result.

6.3 Methods used

All laboratories used the same methods, i.e. UV spectrophotometry and dPCR, for DNA mass concentration measurements and DNA copy number concentration measurements, respectively (Section 1.3).

All types of instruments used during the characterisation study are summarised in Annex G. The laboratory code (e.g. L1) is a random number and does not correspond to the order of laboratories in Section 0. When a laboratory applied 2 methods, the method-code is added to the laboratory code to specify the method used for a dataset (e.g. L1-UV).

6.4 Evaluation of results

The characterisation study resulted in 7 datasets for UV spectrophotometry and in 5 datasets for dPCR. The technical and statistical evaluations of these datasets were performed by IRMM. All individual results of the participants, grouped per method, are displayed in a tabular form and in a graph in Annex H.

6.4.1 Technical evaluation

The data were first assessed for compliance with the analysis protocol and for their validity based on technical reasons. When excluding results, a technical reason was required. The following criteria were considered during the evaluation for UV spectrophotometry and dPCR.

UV spectrophotometry:

- compliance with the analysis protocol: sample preparations and measurements of replicates performed randomly and on different days
- correctness of the dilutions steps: all dilutions steps had to be prepared gravimetrically and masses had to be recorded
- correctness of preparing the blank solution
- correct setting of the baseline
- RSD between replicates over the 6 vials on one day should be below 5 %

dPCR:

- compliance with the analysis protocol: sample preparations and measurements of replicates performed randomly, on different days and on different arrays
- correctness of the dilutions steps: all dilutions steps had to be prepared gravimetrically and masses had to be recorded
- homogeneous distribution of digested lambda DNA over a panel
- RSD between replicates of 1 dilution series over the 6 vials should be below 15 %

Based on the above criteria, the following datasets were rejected as not technically valid (Table 7).

Table 7: Datasets that showed non-compliances with the analysis protocol and technical specifications, and action taken.

Measurement method	Lab-method code	Description of problem	Action taken
UV spectrophotometry	L6-UV	Technical problem with one of the replicates (reported by laboratory)	Result of this replicate was not used
UV spectrophotometry	L7-UV	Technical problem (baseline was not correctly set, resulting in an absorbance shift in the measurements of the 2 days)	Not used for evaluation
dPCR	L5-dPCR	Technical problem (RSD > 15 % in 1 dilution series)	Results of 1 dilution series was not used for evaluation

6.4.2 Statistical evaluation

The valid datasets were assessed for normality of dataset means using kurtosis/skewness tests and normal probability plots. They were assessed 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 8a & 8b.

Table 8a: Statistical evaluation of the technically valid datasets for UV spectrophotometry. *p*: number of technically valid datasets

UV spectrophotometry	*P*	Statistical parameters			
		Mean[1)] [ng/µL]	*s* [ng/µL]	$s_{between}$ [ng/µL]	s_{within} [ng/µL]
ERM-AD442k	6	57.53	0.98	0.95	0.59

[1)] including less than 0.02 ng/µL of non-lambda DNA (i.e. *E. coli* DNA).

Table 8b: Statistical evaluation of the technically valid datasets for dPCR. *p*: number of technically valid datasets

dPCR	*p*	Statistical parameters			
		Mean [cp/µL]	*s* [cp/µL]	$s_{between}$ [cp/µL]	s_{within} [cp/µL]
ERM-AD442k	5	1.20×10^9	1.43×10^8	1.41×10^8	4.82×10^7

The laboratory means follow a normal distribution. None of the data contains outlying means and variances. The datasets were therefore consistent and the mean of laboratory means was a good estimate of the true value.

The uncertainty relating to the characterisation is estimated as the standard error of the mean of laboratory means (Table 9a & 9b).

Table 9a: Uncertainty of characterisation for UV spectrophotometry.

UV spectrophotometry	*p*	Mean[1)] [ng/µL]	*s* [ng/µL]	u_{char} [ng/µL]	$u_{char,rel}$ [%]
ERM-AD442k	6	57.53	0.98	0.40	0.69

[1)] including less than 0.02 ng/µL of non-lambda DNA (i.e. *E. coli* DNA).

Table 9b: Uncertainty of characterisation for dPCR.

dPCR	*p*	Mean [cp/µL]	*s* [cp/µL]	u_{char} [cp/µL]	$u_{char,rel}$ [%]
ERM-AD442k	5	1.20×10^9	1.43×10^8	6.36×10^7	5.32

7 Value Assignment

Certified and indicative values were assigned.

Certified values are values that fulfil the highest standards of accuracy. Procedures at IRMM generally require pooling of not less than 6 datasets to assign certified values. Full uncertainty budgets in accordance with the 'Guide to the Expression of Uncertainty in Measurement' [4] were established. ERM-AD422k was certified for its DNA mass concentration by UV spectrometry.

Indicative values are values where either the uncertainty is deemed too large or where too few independent datasets (i.e. less than 6 datasets) were available to allow certification. In the case of ERM-AD442k only 5 valid datasets for dPCR were obtained and the estimated uncertainty for the copy number concentration measurements found to be too large for certification. Consequently an indicative value was given. Uncertainties were evaluated according to the same rules as for the certified value.

7.1 Certified value and its uncertainty

The unweighted mean of the means of the accepted datasets as shown in Table 9a was assigned as certified value.

The assigned uncertainty consists of uncertainties relating to characterisation, u_{char} (Section 0.4), to potential between-unit inhomogeneity, u_{bb} (Section 4.1), to potential degradation during transport, u_{sts} (Section 5.3), and to potential degradation during long-term storage, u_{lts} (Section 0.3).

The uncertainty relating to degradation during transport (u_{sts}) was not considered in the combined uncertainty, as the samples will be transported on dry ice, as the storage temperature is -20 °C ± 5 °C and as this uncertainty is considered as negligible (i.e. less than one third of the highest uncertainty contribution) in accordance with the Guide to the Expression of Uncertainty in Measurement (GUM) [4].

The uncertainty of the long-term stability (u_{lts}) has been considered in the combined uncertainty. These different contributions were combined to estimate the relative expanded uncertainty of the certified value ($U_{CRM,\,rel}$) with a coverage factor k as:

$$U_{CRM,rel} = k \cdot \sqrt{u_{char,rel}^2 + u_{bb,rel}^2 + u_{lts,rel}^2} \qquad \textbf{Equation 15}$$

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

Because of the sufficient numbers of the degrees of freedom of the different uncertainty contributions for UV spectrophotometry, a coverage factor k of 2 was applied, to obtain the expanded uncertainty. The certified value and its uncertainty are summarised in Table 10.

Table 10: Certified value and its uncertainty for ERM-AD442k using UV spectrophotometry.

	Certified value[1)] [ng/µL]	$u_{char, rel}$ [%]	$u_{bb, rel}$ [%]	$u_{lts, rel}$ [%]	$U_{CRM, rel}$ [%]	U_{CRM} [2)] [ng/µL]
Lambda DNA	57.5	0.69	0.58	0.23	1.85	1.1

[1)] including less than 0.02 ng/µL of non-lambda DNA (i.e. *E. coli* DNA)
[2)] expanded (k = 2) and rounded uncertainty

As described in the purity assessment Section (i.e. Section 3.3.2.3), a negligible percentage of the host DNA (i.e. 0.13 %) was found by qPCR using *E. coli*-specific primers and probes. This percentage represents a DNA copy number concentration of host DNA sequences (i.e. 1.57 x 10^6 cp/µL), converted to a DNA mass concentration of 0.02 ng/µL (using Equation 4).

7.2 Indicative value and its uncertainty

An indicative value was assigned for the DNA copy number concentration using dPCR as the expanded combined uncertainty is rather large and because the number of valid datasets is below 6. However, the results were regarded as sufficiently trustworthy to assign as an indicative value. An indicative value may not be used as a certified value. The uncertainty budget was calculated as for the certified value and is listed together with the assigned value in Table 11.

For dPCR, the effective number of degrees of freedom was calculated to be 7, using the Welch-Sattertwaithe equation in accordance with the Guide to the Expression of Uncertainty in Measurement (GUM) [4], and correspondingly a factor k of 2.36 was chosen.

Table 11: Indicative value and its uncertainty for ERM-AD442k using dPCR.

	Indicative value [cp/µL]	$u_{char, rel}$ [%]	$u_{bb, rel}$ [%]	$u_{lts, rel}$ [%]	$U_{CRM, rel}$ [%]	U_{CRM} [1)] [cp/µL]
Lambda DNA	1.20 x 10^9	5.32	1.75	1.99	14.01	0.17 x 10^9

[1)] expanded (k = 2.36) and rounded uncertainty

Using Equation 4 the indicative value for DNA copy number concentration determined by dPCR is converted into DNA mass concentration, the indicative value for DNA mass concentration using dPCR is 59.54 ng/µL and the U_{CRM} is 8.34 ng/µL.

Another dPCR assay (named as lambda DNA assay 2 in Annex Table A4) was performed targeting another region of the lambda DNA sequence. This DNA sequence region has the same GC content as the DNA sequence region that was targeted by primers and probe given in Annex Table A1 (which is the same as lambda DNA assay 1 in Annex Table A4). The same restriction enzyme was used which is *Taq(α)I*. One vial was analysed according to Section 1.3.1 and having 5 replicates. The results were analysed using ERM Application Note 1 (www.erm-crm.org, [27]), revealing no bias between the measurements of lambda DNA assay 2 and the indicative value of the DNA copy number concentration by dPCR.

8 Metrological traceability and applicability of ERM-AD442k

8.1 Metrological traceability

Identity

Identity was confirmed by two expert sequencing service companies using two independent NGS methods: i.e. an Illumina platform based on base-specific fluorescence and a GS Junior platform (Roche) based on pyrosequencing. NGS methods are high-throughput and deep sequencing methods, where multiple DNA fragments are sequenced simultaneously and in multiple times. The participants used different methods for the sample preparation as well as for the final determination, demonstrating absence of measurement bias. The identity of the measurand is therefore structurally defined and independent of the measurement method.

Quantity value

The DNA mass concentration of ERM-AD442k is a method-defined measurand and has been obtained by UV spectrophotometry. The DNA copy number concentration of ERM-AD442k is a method-defined measurand and has been obtained by dPCR using primers, probe and PCR conditions shown in Annex Table A1 – A3. Traceability of the results is based on the traceability of all relevant input factors. Instruments in individual laboratories were verified and calibrated with tools ensuring traceability to the SI. Consistency within each of the two inter-laboratory comparisons demonstrates that all relevant input factors were covered. As the assigned values are combinations of agreeing results, individually traceable to the SI, the assigned quantity values themselves are also traceable to the SI.

In conclusion, the measurand is method-defined as the certified value was measured by UV spectrophotometry using an absorption coefficient for denatured DNA of 37 ng/µL [19] in Equation 1 and as the indicative value was measured by digital PCR.

8.2 Applicability of ERM-AD442k

The applicability of a CRM defines its fitness for use. When applicability of a CRM is not established, the results from routinely used applications cannot be legitimately compared with the certified value to determine whether a bias does not exist in calibration, nor can the CRM be used as a calibrant.

ERM-AD442k is prepared from lambda DNA and the analytical behaviour will be the same as for a routine sample of lambda DNA. For other types of samples other than lambda DNA the applicability has to be assessed.

This CRM is mainly used as a calibrant to set up calibration curves. Ideally, a calibration curve contains several different concentrations by diluting the CRM to the desired concentrations. A fit-for-purpose test was performed to compare calibration curves prepared with ERM-AD442k (with certified DNA mass concentration) and with a commercial lambda DNA (with a stated DNA mass concentration) using a Quant-iT^{TM} PicoGreen dsDNA assay (Life Technologies). From a lambda DNA solution with a DNA mass concentration of 2 ng/µL, 5 final DNA mass concentrations (0.10 ng/µL, 0.25 ng/µL, 0.50 ng/µL, 0.75 ng/µL and 0.90 ng/µL) were prepared and used in duplicate to construct one calibration curve. Four

independent calibration curves were prepared with the ERM-AD442k as well as with the commercial lambda DNA. The results of the lambda DNA CRM and the commercial lambda DNA are compiled in Annex I. The coefficient of determination of the linear regression (r^2) is 0.9902 for the calibration curve of ERM-AD442k and 0.9900 for the calibration curve of the commercial lambda DNA. Both results are therefore comparable. The Quant-iT^{TM} PicoGreen dsDNA assay demonstrated that the ERM-AD442k lambda DNA in solution is suitable for using as a calibrant, while applying the certified DNA mass concentration value of the UV spectrophotometry.

9 Instructions for use

9.1 Safety information

The usual laboratory safety measures apply.

9.2 Storage conditions

The materials shall be stored at -20 °C ± 5 °C in the dark.

Providing microbial contamination was excluded during the production, the lambda DNA in solution can be used for numerous experiments. As outlined in the Certification Report, the material should not exceed more than 5 freeze-thaw cycles to avoid inconsistency in the DNA mass concentrations. The material can also be stored at 4 °C for 4 weeks as changes to the certified DNA mass concentration observed during that period are not significant. It is advisable to close the vial with the original screw cap after use. The lambda DNA in solution should not be exposed to direct sun light or any other UV radiation.

Please note that the European Commission cannot be held responsible for changes that happen during storage of the material at the customer's premises, especially of opened vials.

9.3 Preparation and use of the material

When the vial of lambda DNA solution is used for the first time, the material should be prepared as follows. Thaw the contents of the vial by leaving the vial at 4 °C overnight. Bring the vial and its contents to room temperature followed by homogenising the contents of the vial thoroughly and gently for 10 min. This homogenising process needs to be repeated once more.

It is recommended not to freeze-thaw ERM-AD442k more than 5 times in order to have consistency in the DNA mass concentration. Therefore, aliquoting ERM-AD442k is highly recommended after the first thaw and after homogenising thoroughly.

The vials should be opened and handled under a laminar flow to reduce the risk of contamination.

9.4 Minimum sample intake

The minimum sample intake is 50 µL for UV spectrophotometry and 68 µL for dPCR.

9.5 Use of the certified value

The main purpose of the material is to use as a calibrant. The material and its certified value are intended to be used for the calibration of DNA quantification methods, quality control and assessment of method performance, i.e. for checking accuracy of analytical results or calibration, since this lambda DNA calibrant has a wide and versatile use. As any reference material, it can also be used for control charts or validation studies.

Use as a calibrant

It is recommended to use this material as calibrant. The uncertainty of the certified value shall be taken into account in the estimation of the measurement uncertainty.

Comparing an analytical 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, www.erm-crm.org [27]).

For assessing the method performance, the measured values of the CRMs are compared with the certified values. The procedure is described here in brief:

- Calculate the absolute difference between mean measured value and the certified value (Δ_{meas}).
- Combine 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 $\Delta_{meas} \leq U_{\Delta}$ no significant difference exists between the measurement result and the certified value, at a confidence level of about 95 %.

Use for quality control and in quality control charts

The materials can be used for quality control charts and also as a quality control material. All vials can be treated as containing the same concentration of solution as a contribution has been included in the combined uncertainty to represent any between-vial inhomogeneity.

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Annexes

Annex A: Description of the (d)qPCR technique

The DNA copy number concentration of lambda DNA in ERM-AD442k has been determined by dPCR using the protocol described below. After the lambda DNA in each ERM-AD442k vial was digested with the restriction enzyme *Taq(α)I*, two independent DNA dilution series were prepared gravimetrically. For each dilution series, 25 µL of digested ERM-AD442k was gravimetrically diluted with $T_1E_{0.01}$ buffer to obtain DNA samples at a nominal final DNA copy number concentration of 447 cp/µL. A volume of 3.54 µL of this diluted digested DNA sample was further mixed with 5.46 µL pre-sample mix solution. This mixture, having a total volume of 9 µL, was loaded in 1 panel of the BioMark™ 12.765 digital arrays. The pre-sample mix contained 20x GE sample loading reagent (Fluidigm) and 2x TaqMan Universal PCR MasterMix (Applied BioSystems), of which the concentration was used according to the manufacturer. The concentration of the primers and probe in the pre-sample mix are given in Table A1. The dPCR was performed according to the condition specifications in Table A2. The dPCR runs were analysed with the Fluidigm Digital PCR Analysis software using either the parameters in Table A3 or manual calling.

Table A1. Primers and probe used to amplify the lambda DNA fragment in a simplex dPCR. FAM: fluorescein amidite. BHQ1: Black Hole Quencher 1. *Reference sequence is the complete genome of *phage lambda* NC_001416.1

Primer/ probe	Coordinates in reference sequence*	Sequence (5' to 3')	Concentration in PCR [nM]	Amplicon size [bp]
Forward primer	5845…5863	TGCAATGACCCCGCTGATG	500	147
Reverse primer	5931…5955	CGGAACGTGCCGGACTTG	500	
Probe	5974…5991	6-FAM-CTGGTCTGGTCAGCAGCAACCGCAA-BHQ1	100	

Table A2. Thermal cycling protocol used for the simplex dPCR amplifying lambda DNA target.

Name	Phase	Time [seconds]	Temperature [°C]	Repeats
Uracil-N-glycosylase (UNG) and Hot start	UNG	120	50	1
	Hot start	600	95	
PCR cycle	Denaturation	15	95	45
	Annealing	60	60	
Image should be captured after the end of each cycle.				

Table A3. Digital PCR analysis parameters

Quality threshold	0.05
Baseline Correction	linear
Ct threshold method	Auto (Global)
Target Ct Range	10-45

The purity assessment of ERM-AD442k was performed by NGS and qPCR. For the latter, ABI Prism 7900HT (Applied BioSystems) was used. Table A4 shows the primers and probes used for the quantification of the host (*E. coli*) DNA fragments. Brilliant II QPCR Master Mix (Agilent, Diegem, BE) was used according to the manufacturer. The PCR conditions were the same as described in Table A2. The qPCR runs were analysed with the Sequence Detection Systems software version 2.4.1 (Applied BioSystems).

Table A4. Primers and probe used to amplify a lambda DNA fragment and an *E. coli* DNA fragment in a simplex qPCR. FAM: fluorescein amidite. BHQ1: Black Hole Quencher 1. HEX: hexachlorofluorescein.

Primer/probe	Sequence (5' to 3')	Concentration in PCR [nM]	Amplicon size [bp]
Lambda DNA assay 1			
Forward primer	TGCAATGACCCCGCTGATG	500	147
Reverse primer	CGGAACGTGCCGGACTTG	500	
Probe	6-FAM-CTGGTCTGGTCAGCAGCAACCGCAA-BHQ1	100	
Lambda DNA assay 2			
Forward primer	TGCGCTGTATGCCGGTATG	500	188
Reverse primer	GTTGTTCGGGTCAATCCAGTTC	500	
Probe	5-HEX-CCTCAACGGCATTATGGCGGTCCTT-BHQ1	300	
E. coli assay 1			
Forward primer	CCATCTTCCAGCCCCTCTTC	900	74
Reverse primer	CTGAATTCCGCTTTTAAACCTTCT	900	
Probe	6-FAM- ACGTCGAATCGCCTCTGGCTGATC-BHQ1	200	
E. coli assay 2			
Forward primer	GCGAACCGTAGCCATCCA	900	75
Reverse primer	AACTGCATGCGGCACTACTG	900	
Probe	6-FAM-TTCCGCTTTAACCCCGCGTTGC-BHQ1	200	

Annex B: Results of the homogeneity measurements

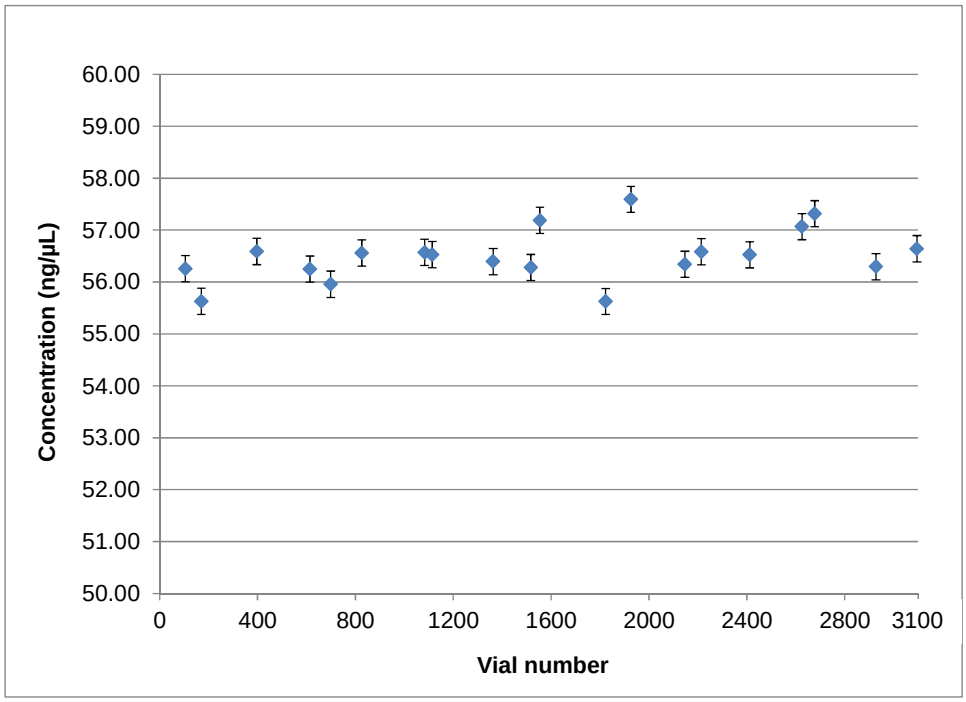

Figure B1. Homogeneity study using UV spectrophotometry for DNA mass concentration in ERM-AD442k: the vial mean ± the confidence interval of the means (calculated according to Equation 16).

$$CI_{means} = t_{95\%;2} * \frac{s_{wb}}{\sqrt{n}}$$ **Equation 16**

CI_{means}	confidence interval of the means
n	number of vials analysed
$t_{95\%;2}$	Student value for degrees of freedom of s_{wb} and a confidence level of 95%
s_{wb}	standard deviation within groups from one way ANOVA for all vials

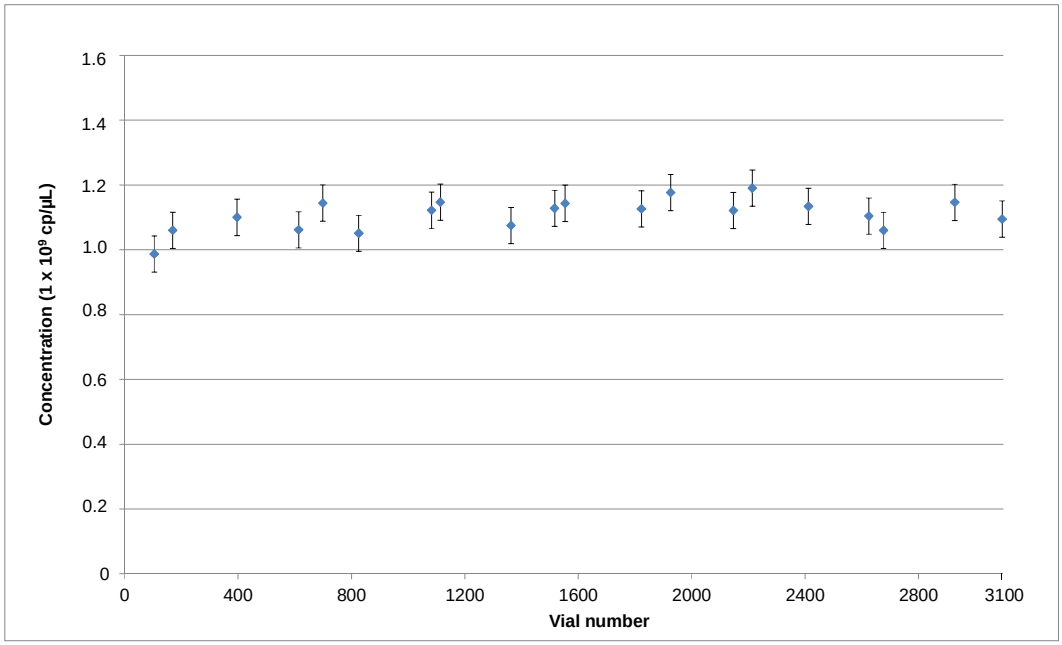

Figure B2. Homogeneity study using dPCR for DNA copy number concentration in ERM-AD442k: the vial mean ± the confidence interval of the means (calculated according to Equation 16).

Annex C: Results of the minimum sample intake measurements

Table C1: Results of the statistical evaluation of the minimum sample intake study for UV spectrophotometry at a 95 % confidence level

Sample intake [µL]	Measurement results [ng/µL]	Standard deviation [ng/µL]	Variances [ng/µL]
5	52.43	2.60	6.74
10	55.73	2.31	5.34
15	56.46	1.64	2.70
20	56.83	1.19	1.41
50	56.23	0.39	0.15
100	57.16	0.22	0.05

Annex D: Results of the short-term stability measurements

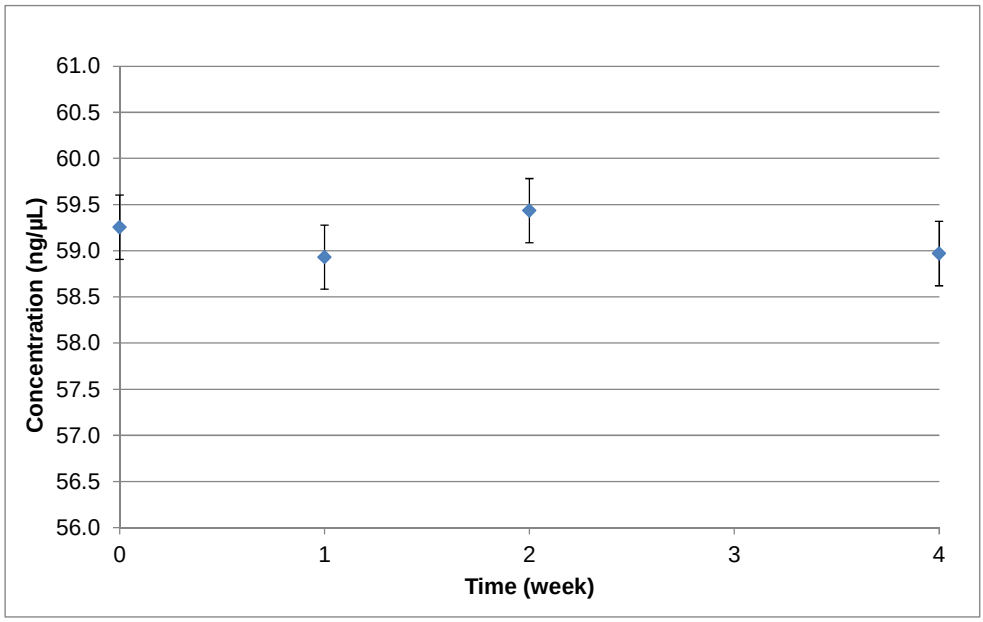

Figure D1. Short-term stability study at -20 °C using UV spectrophotometry for DNA mass concentration in ERM-AD442k.

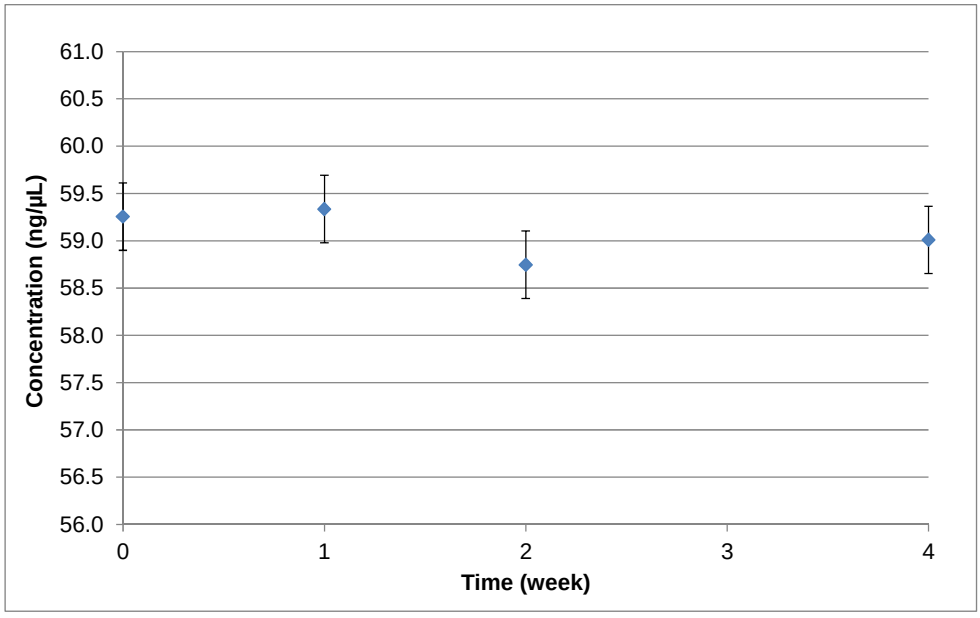

Figure D2. Short-term stability study at 4 °C using UV spectrophotometry for DNA mass concentration in ERM-AD442k.

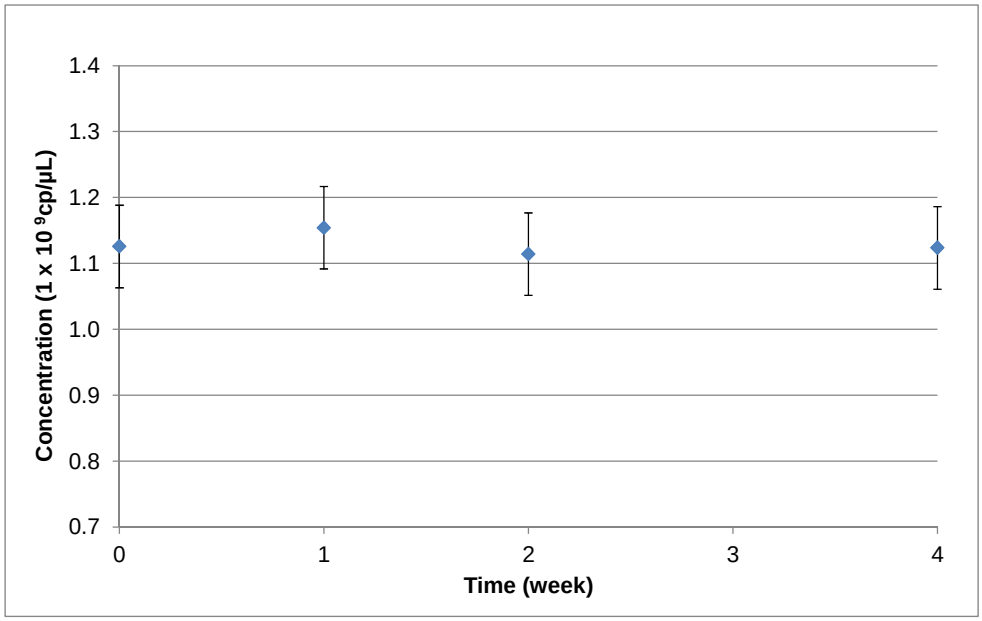

Figure D3. Short-term stability study at -20 °C using dPCR for DNA copy number concentration in ERM-AD442k.

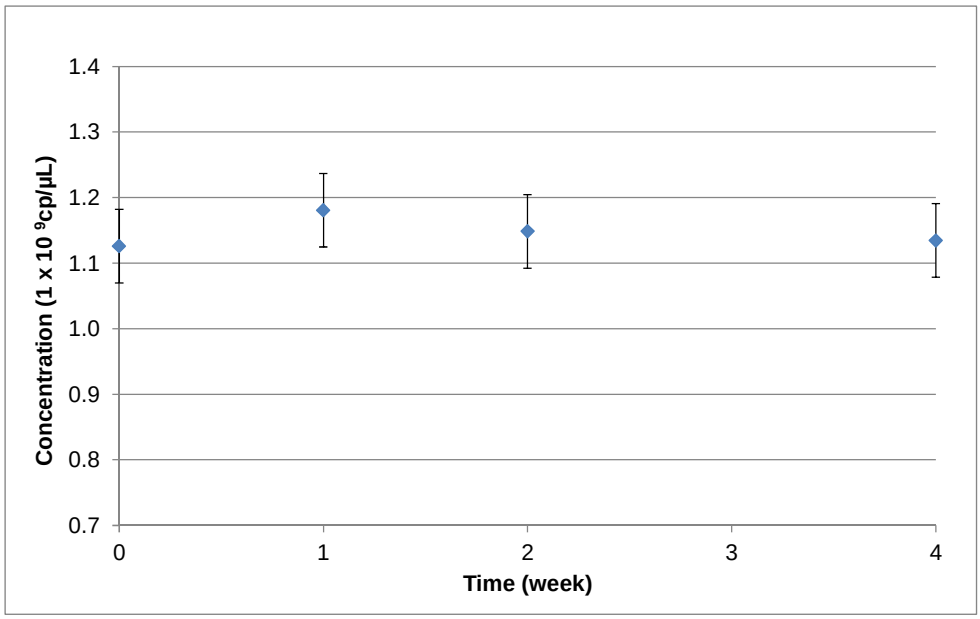

Figure D4. Short-term stability study at 4 °C using dPCR for DNA copy number concentration in ERM-AD442k.

Annex E: Results of the long-term stability measurements

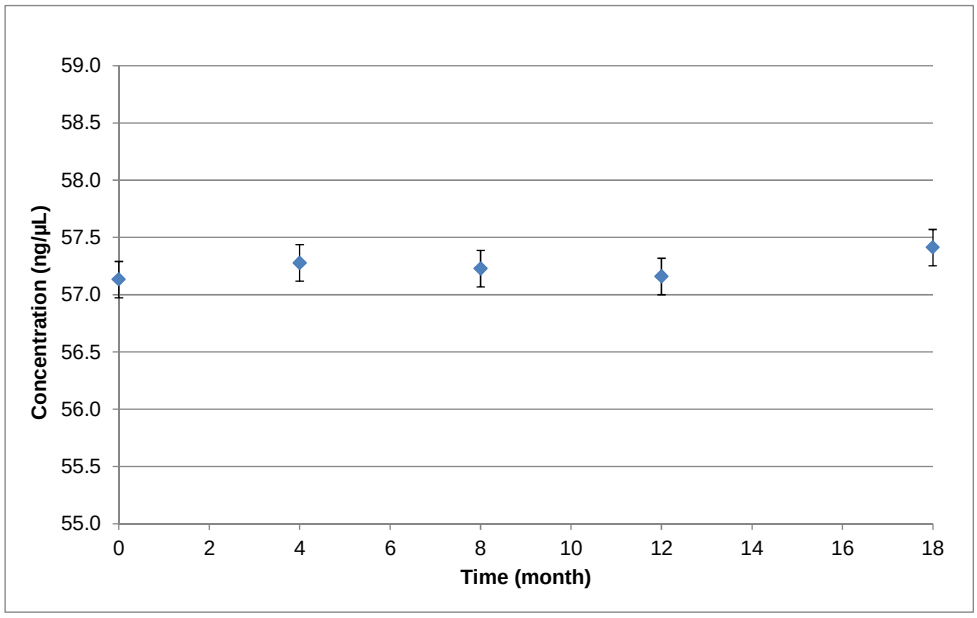

Figure E1. Long-term stability study at -20 °C using UV spectrophotometry for DNA mass concentration in ERM-AD442k.

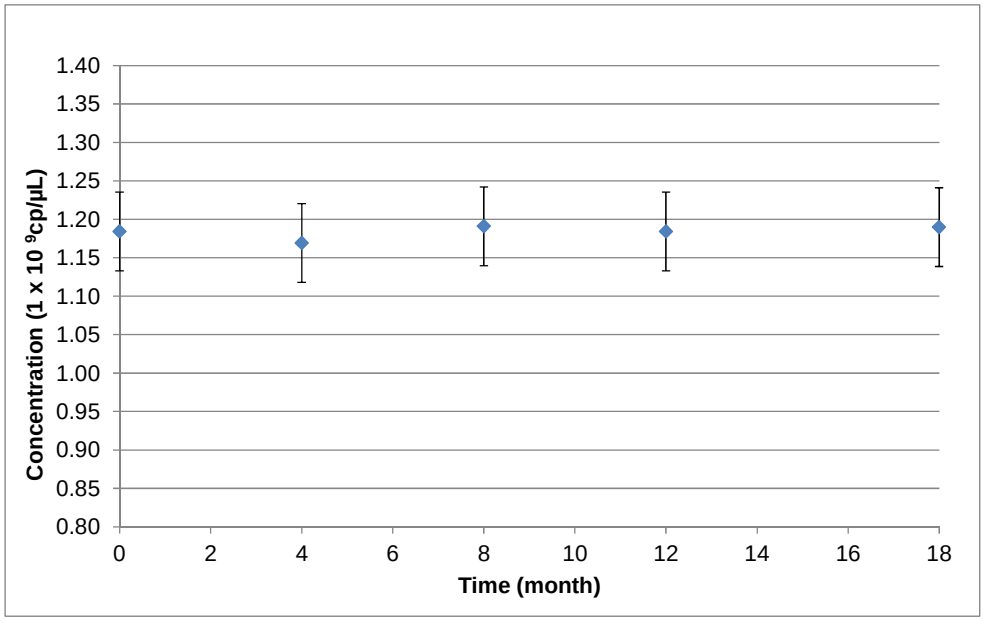

Figure E2. Long-term stability study at -20 °C using dPCR for DNA copy number concentration in ERM-AD442k.

Annex F: Results of the freeze-thaw study

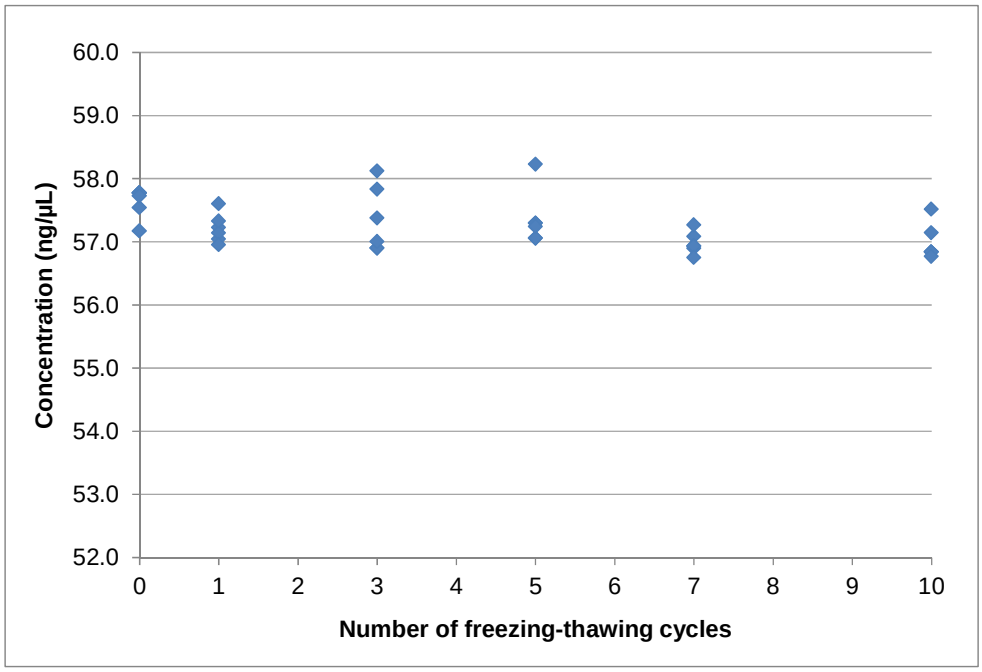

Figure F. UV spectrophotometrical analyse of the consistency of DNA mass concentration in ERM-AD442k over 10 freeze-thaw cycles.

Annex G: Summary of methods used in the characterisation study

Table G. Summary of instruments used in the characterisation study per laboratory. n.a.: not applicable.

Laboratory	Instrument used for	
	UV spectrophotometry	dPCR
L1	BioPhotometer (Eppendorf)	BioMark™ HD System (Fluidigm)
L2	UV-2450 UV-Vis spectrophotometer (Shimadzu)	
L3	Biotek Synergy MX (Biotek)	
L4	Cary 4000 UV-Vis spectrophotometer (Agilent)	
L5	Infinite 200 (Tecan)	
L6	8453 DAD-UV spectrophotometer (Agilent)	n.a.
L7	UV/Vis spectrophotometer UV2 (Unicam)	n.a.

Annex H: Results of the characterisation measurements

Table H1. Results of the characterisation study of the DNA mass concentration in ERM-AD442k using UV spectrophotometry

Laboratory number	DNA mass concentration [ng/µL]						Mean	Uncertainty (k = 2)
	Vial 1	Vial 2	Vial 3	Vial 4	Vial 5	Vial 6	[ng/µL]	[ng/µL]
L1	55.42	58.04	57.58	56.94	57.47	57.37	57.02	0.34
L2	58.29	58.88	57.31	59.09	58.87	58.75	57.07	0.29
L3	56.76	56.95	56.79	56.87	57.53	57.54	58.94	0.48
L4	59.21	59.18	59.27	59.59	58.15	58.26	57.14	0.75
L5	56.56	56.88	56.53	57.29	57.32	57.52	56.46	0.32
L6	57.03	56.36	55.95	56.54	56.72	56.15	58.53	0.54
Results not used for certification								
L7	51.87	53.20	52.20	53.20	52.67	52.15	52.55	0.46

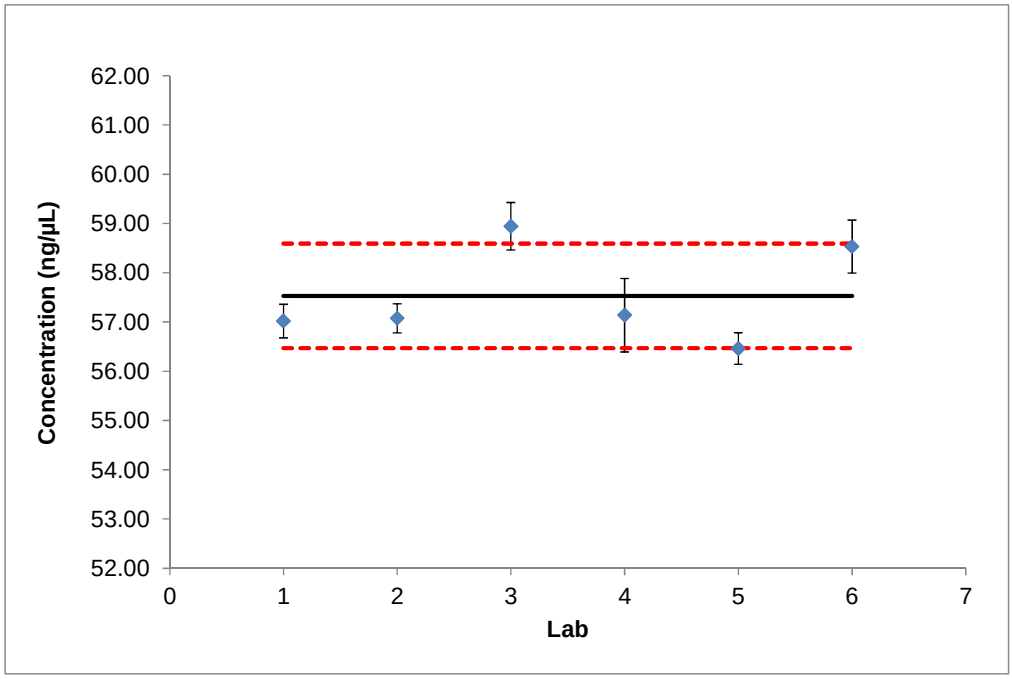

Figure H1. Results of the characterisation study of the DNA mass concentration in ERM-AD442k using UV spectrophotometry. The error bars represent expanded uncertainties. The solid line represents the certified value (the mean of laboratory means), while the broken lines represent the expanded uncertainty of the certified value.

Table H2. Results of the characterisation study of the DNA copy number concentration in ERM-AD442k using dPCR

Laboratory number	DNA copy number concentration [cp/µL]						Mean	Uncertainty (k = 2)
	Vial 1	[ng/µL]	[ng/µL]	Vial 4	Vial 5	Vial 6	[cp/µL]	[cp/µL]
L1	1.25×10^9	1.31×10^9	1.38×10^9	1.43×10^9	1.40×10^9	1.37×10^9	1.36×10^9	5.09×10^7
L2	1.01×10^9	1.00×10^9	1.00×10^9	1.01×10^9	0.99×10^9	0.95×10^9	0.99×10^9	1.73×10^7
L3	1.20×10^9	1.33×10^9	1.33×10^9	1.32×10^9	1.32×10^9	1.31×10^9	1.30×10^9	3.90×10^7
L4	1.19×10^9	1.23×10^9	1.15×10^9	1.19×10^9	1.18×10^9	1.19×10^9	1.19×10^9	2.25×10^7
L5	1.15×10^9	1.19×10^9	1.10×10^9	1.17×10^9	1.22×10^9	1.04×10^9	1.15×10^9	5.31×10^7

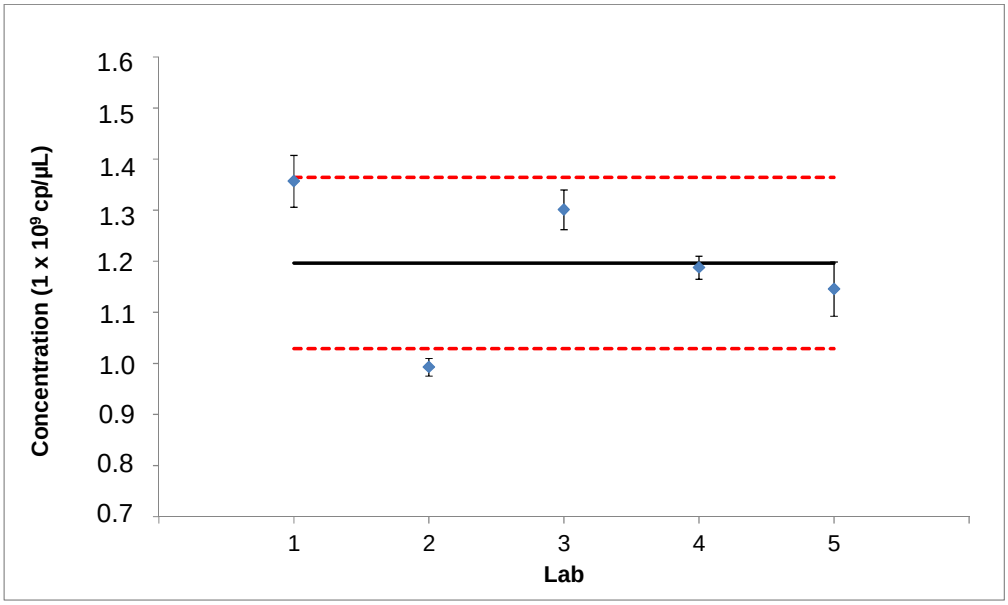

Figure H2. Results of the characterisation study of the DNA copy number concentration in ERM-AD442k using dPCR. The error bars represent expanded uncertainties. The solid line represents the indicative value (the mean of laboratory means), while the broken lines represent the expanded uncertainty of the indicative value.

Annex I: Applicability of CRM

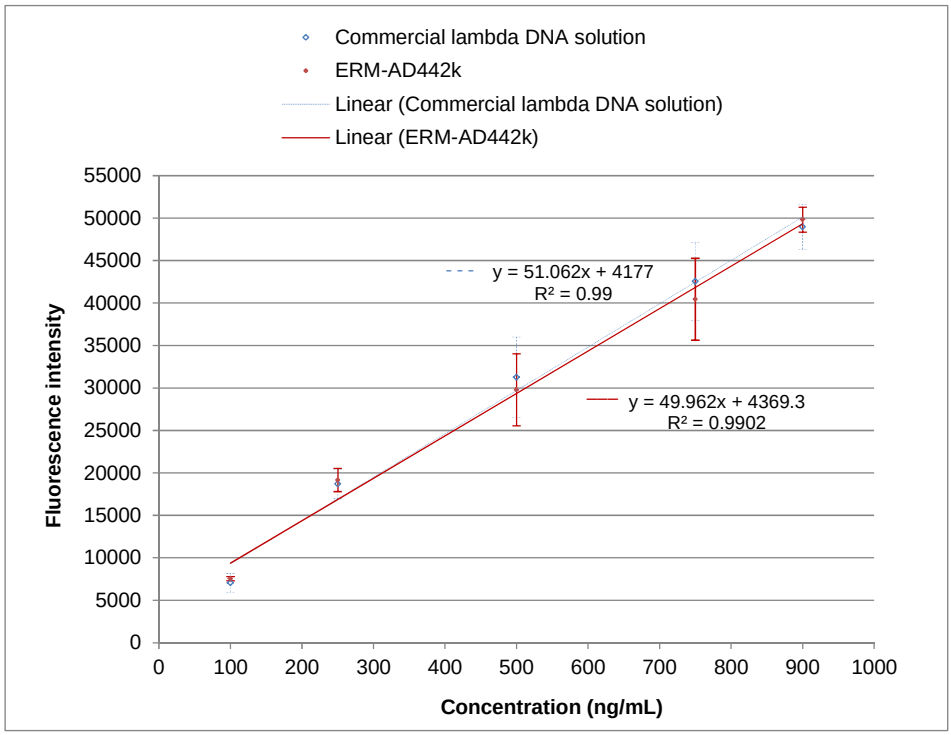

Figure I1. Comparable results between ERM-AD442k and a commercial lambda DNA solution using Quant-iT^{TM} PicoGreen dsDNA assay. The solid line is the regression line of ERM-AD442k and the dashed line is the regression line of the commercial lambda DNA solution. The solid error bars are the standard deviations of ERM-AD442k and the dashed error bars are the standard deviations of the commercial lambda DNA solution.

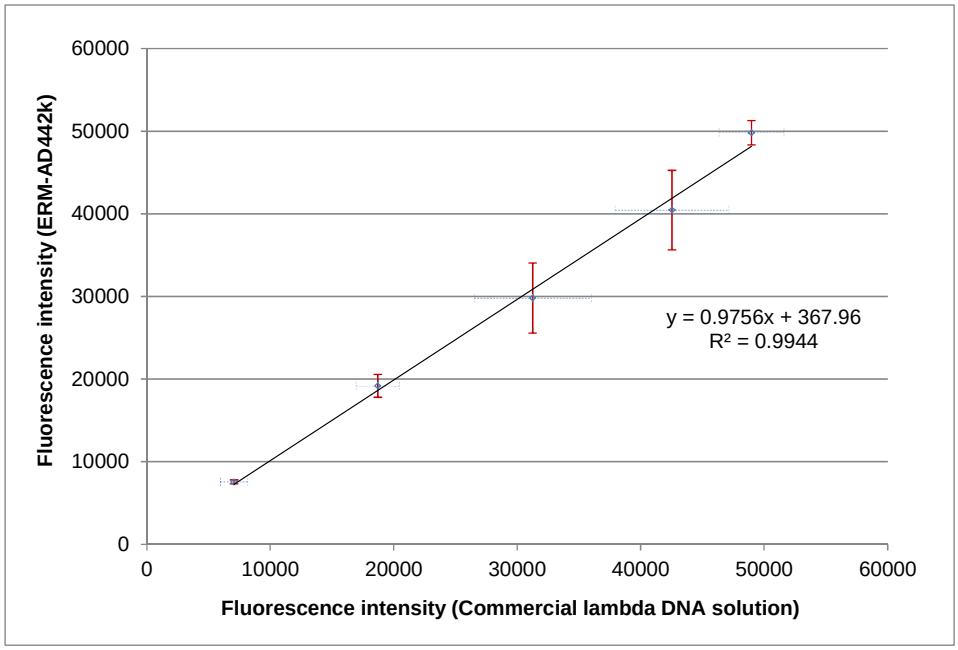

Figure I2. Comparable results between ERM-AD442k and a commercial lambda DNA solution using Quant-iT^{TM} PicoGreen dsDNA assay. The solid error bars are the standard deviations of ERM-AD442k and the dashed error bars are the standard deviations of the commercial lambda DNA solution.

European Commission

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Certified Reference Material: ERM®-AD442k

Author(s): Pui Yan Jenny Chung, Philippe Corbisier, Stéphane Mazoua, Ingrid Zegers, Guy Auclair, Stefanie Trapmann, Hendrik Emons

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