EUROPEAN COMMISSION
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Institute for Reference Materials and Measurements

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IRMM-448

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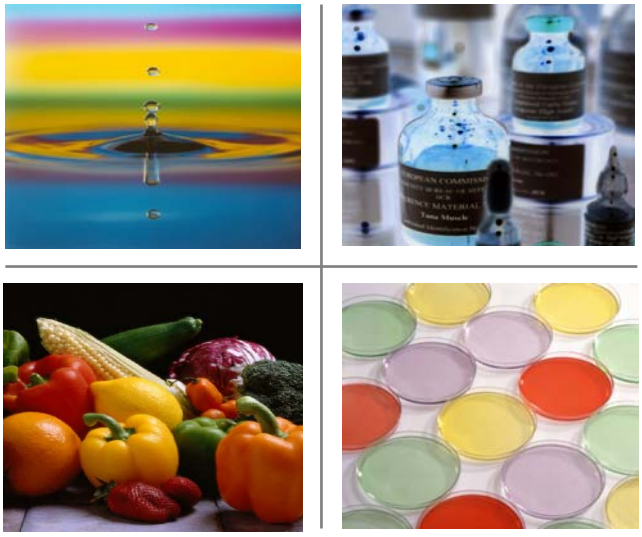

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SUMMARY

This report describes the production and certification of a reference material of purified genomic DNA (gDNA) of *Campylobacter jejuni* (NCTC 11351). This CRM (IRMM-448) supports the harmonisation and validation of PCR procedures by their use as taxonomic controls in PCR reactions. The homogeneity and stability (at three different temperatures for up to 4 weeks) of the batch was assessed by monitoring the amplification of the *ceu*E gene by PCR, by agarose gel electrophoresis of gDNA and determination of the mass of gDNA per vial. The material is stable at 4 and 18 °C. The identity of the gDNA was confirmed by DNA sequence analysis of the *ceu*E gene. Each vial contains a certified mass of 71 ng freeze-dried gDNA, with an expanded uncertainty (U_{CRM}) of 39 ng (k = 2).

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GLOSSARY

a	intercept of the regression line
ANOVA	analysis of variance
b	slope of the regression line
bp	base pairs
C.	*Campylobacter*
CV	coefficient of variation
DNA	deoxyribo nucleic acid
DTCS	dye terminator cycle sequencing
EDTA	Ethylenediaminetetraacetic acid
dNTP	deoxy nucleotide triphosphates
ds gDNA	double-stranded genomic deoxyribo nucleic acid
MS	average of squares
MS_{among}	average square among vials from an ANOVA
MS_{within}	average square within vials from an ANOVA
N	total number of vials
n	total number of data points
n.a.	not applicable
NCTC	National Collection of Type Cultures
PCR	polymerase chain reaction
s_{bb}	standard deviation between the vials
SS	the sum of the squares of the data (ANOVA)
t	time
$t_{\alpha,\,\nu}$	*t*-value (two-sided) for a confidence level α and ν degrees of freedom
TBE	8.9 mmol/L Tris, 8.9 mmol/L boric acid, 0.2 mmol/L EDTA, pH 8.3
TE	10 mmol/L Tris, 1 mmol/L EDTA, at specified pH
Tris	Tris(hydroxymethyl)aminomethane
u_b	standard uncertainty of the slope of a regression line
u^*_{bb}	uncertainty component due to the inhomogeneity that can be hidden by the repeatability of the procedure

u_{char}	standard uncertainty contribution of characterisation
U_{CRM}	certified expanded uncertainty
$u_{rel,\ lts}$	relative uncertainty contribution of long-term stability study
$u_{rel,\ sts}$	relative uncertainty contribution of short-term stability study
ν	degrees of freedom
$\overline{x}$	average of all *x*-values
$\overline{y}$	average of all *y*-values

1 Introduction

Stable genomic DNA (gDNA) standards have been developed previously for the verification and detection of food-borne pathogens by diagnostic polymerase chain reaction (PCR) within the European FOOD-PCR project [1 - 4]. These standards support harmonisation and validation of different PCR procedures by their use as taxonomic controls in PCR reactions.

This report describes the production and certification of a batch of 718 vials of purified gDNA from *Campylobacter jejuni* (NCTC 11351). The certification of IRMM-448 was performed according to IRMM RM Unit procedures applying a quality management system according to ISO Guide 34 [5 - 8]. Short-term stability measurements were performed at three different temperatures (4, 18 and 60 °C) in the frame of the certification project. The produced batch was characterised for homogeneity and with respect to the determination of the mass of gDNA per vial with a calculated expanded uncertainty (U_{CRM}).

The *ceu*E gene of *Campylobacter jejuni* encodes for a 34.5 to 36.2 kDa lipoprotein component of the binding-protein-dependent transport system for enterochelin (a siderophore) [9]. The DNA sequence of *ceu*E is characteristic for *Campylobacter jejuni* and can be used for its detection, identification and discrimination from other bacterial strains [9]. Contents of vials used in stability and homogeneity measurements were analysed by amplification of a part of the *ceu*E gene using qualitative PCR. Identification of *Campylobacter jejuni* was performed by DNA sequence analysis of the *ceu*E gene.

2 Participants

The production and certification of IRMM-448 was performed by IRMM. A summary of the different steps in the development, processing and certification of IRMM-448 is given in Table 1.

Table 1. Summary of different steps in the development, processing and certification of IRMM-448.

Time	Processing step
May – June 2006	Growth of *C. jejuni* and isolation of gDNA
June – July 2006	Feasibility study
Aug. 2006	Processing of IRMM-448
Sept – Oct. 2006	Certification
Oct. 2006	Certification report and certificate

3 Processing steps

3.1 Growth of C. jejuni

C. jejuni (NCTC 11351) was grown on Mueller-Hinton agar in an anaerobic jar at 42 °C under a microaerobic atmosphere generated with Anaerocult® C (Merck KgaA, Darmstadt, DE). Liquid cultures were grown in Mueller-Hinton broth in anaerobic jars on a shaker at 60 rpm at 42 °C using the same microaerobic atmosphere.

3.2 Isolation of gDNA

Genomic DNA was isolated from 1.5 L total culture volume and purified using QIAGEN Genomic-tips 500/G and Qiagen Genomic DNA Buffer Set (Qiagen, Hilden, DE) [10]. gDNA was stored as a concentrated solution at -20 ºC in TE, pH 8.0.

3.3 Processing of IRMM-448

1. A solution of mass concentration 2.6 µg purified gDNA/mL TE, pH 8.0 (37 mL total volume) was prepared.
2. The concentration of double-stranded (ds) gDNA was determined by fluorescence of PicoGreen® using lambda phage DNA as external standard [11]. The integrity of gDNA was verified by agarose gel electrophoresis and amplification of the *ceu*E gene by PCR.
3. A test batch was prepared by filling 20 polypropylene vials (sterile, 0.5 mL volume) with 50 µL gDNA solution (mass concentration 2.6 µg/mL TE, pH 8.0).
4. 20 vials freeze-dried gDNA were produced using a Christ Epsilon 2-85D freeze-dryer (Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, DE) using the internal program '15 food-PCR bact DNA'.
5. After freeze-drying, vials were closed with screw caps under argon atmosphere.
6. 10 vials were analysed. The concentration of ds gDNA was determined by fluorescence of PicoGreen® using lambda phage DNA as external standard. The integrity of gDNA was verified by agarose gel electrophoresis. The amplification of the *ceu*E gene by PCR was verified.
7. 718 labeled polypropylene vials (sterile, 0.5 mL volume) were filled with 50 µL gDNA solution (mass concentration 2.6 µg/mL TE buffer, pH 8.0) as in step 3.
8. 718 vials freeze-dried gDNA were produced as in step 4.
9. After freeze-drying vials were closed with screw caps under argon atmosphere.
10. Homogeneity study, stability study, and batch characterisation were carried out.

4 Procedures

Minimum sample volume. A minimum sample intake of 1 μL reconstituted material (2.7 ng gDNA) gives a positive result for qualitative detection of the *ceu*E gene by PCR, and is therefore recommended in PCR reactions [1]. A solution of gDNA was prepared by dissolving the content of one vial in 50 μL TE (pH 8.0), using the procedure described in section 11.2. A sample volume of 10 μL was used for quantification of gDNA by PicoGreen® [11] and 10 μL for agarose gel electrophoresis of gDNA.

Quantification of gDNA. Quantification of ds gDNA in solution was performed with PicoGreen® reagent in a microtiter plate using fluorimetry (excitation at 480 nm and emission at 525 nm) [11]. A standard DNA calibration curve was prepared with lambda phage DNA at mass concentrations ranging from 0 to 1 ng/μL. Fluorescence was detected in a FLUOstar Galaxy plate reader (BMG LABTECH GmbH, Offenburg, DE) [11].

Agarose gel electrophoresis. To 10.0 μL sample, of either gDNA or PCR product, 1.0 μL BlueJuice™ Gel Loading Buffer[1)] (Invitrogen, Merelbeke, BE) was added. From this sample 10 μL was loaded on a mass fraction 0.8 % (for gDNA) or 1.2 % (for PCR products) agarose gel in 0,089 mol/L Tris-borate, 2 mmol/L EDTA, pH 8.3 containing 0.5 μg/mL ethidium bromide (Sigma, St. Louis, US). Electrophoresis was performed at 180 V for 30 min using a Mini-Sub Cell GT electrophoresis system and a Power Pac 3000 (Bio-Rad, Nazareth, BE). Gels were photographed on a GeneScan gel documentation system (Syngene, Cambridge, UK).

PCR. Each reaction mixture for *C. jejuni* specific PCR reactions (25 or 50 µL total volume) contained PCR buffer, 1.5 mmol/L $MgCl_2$, 200 µmol/L dNTP, 1.0 µmol/L JEJ1 primer, 1.0 µmol/L JEJ2 primer, 1 unit Platinum® *Taq* DNA polymerase and 1 μL gDNA template. PCR primers used are summarized in Table 2. All other reaction components for PCR reactions were provided by Invitrogen (Merelbeke, Belgium). PCR reactions were performed using the following time programme: denaturation

[1)] BlueJuice™ Gel Loading Buffer is composed of mass concentration 65 % sucrose, 10 mmol/L Tris-HCl (pH 7.5), 10 mmol/L EDTA, and mass concentration 0.3 % bromophenol blue.

gDNA at 94 ºC for 3 min; 30 cycles denaturation 94 ºC for 30 s, annealing at 57 ºC for 30 s, extension at 72 ºC for 1 min; incubation at 72 ºC for 5 min; hold at 4 ºC until further processing. PCR products were analysed and visualised by agarose gel electrophoresis [1, 2].

Conditions for *E. coli* O157 and *L. monocytogenes* specific PCR reactions (see section 8.2) were used as described previously [3, 4].

Table 2. Primers used for the qualitative PCR.

Organism	PCR target, amplicon length	Primer	DNA sequence	Reference
Campylobacter jejuni (NCTC 11351)	*ceu*E gene, 793 bp	JEJ1 JEJ2	5' CCTGCTACGGTGAAAGTTTTGC 3' 5' GATCTTTTTGTTTTGTGCTGC 3'	[9]
Escherichia coli O157 (EDL933)	fliC gene, 625 bp	fliCh7-F fliCh7-R	5'-GCG CTG TCG AGT TCT ATC GAG C-3' 5'-CAA CGG TGA CTT TAT CGC CAT TCC-3'	[12]
Listeria monocytogenes 4B (NCTC 11994)	listeriolysin O, prfA gene, 274 bp	LIP1 LIP2	5'-GAT ACA GAA ACA TCG GTT GGC-3' 5'-GTG TAA CTT GAT GCC ATC AGG-3'	[13]

DNA sequencing. PCR products (50 µL), amplified with primers JEJ1 and JEJ2, were purified using the QiaQuick® PCR Purification Kit (Qiagen, DE) and cloned into pCR2.1 (Invitrogen, US). Plasmid DNA was purified using the QIAprep Spin Miniprep Kit (Qiagen, DE). Purified plasmid DNA was used in the dye terminator cycle sequencing (DTCS) reaction, according to the DTCS chemistry protocol [15]. Samples were analysed on a CEQ™ 8000 genetic analysis system (Beckman Coulter, Inc., Fullerton, CA, US), using the following procedure: denaturation 120 s at 90 ºC, injection 15 s at 2.0 kV, separation 85 min at 4.2 kV. The correctness of obtained DNA sequence information was confirmed by sequencing and analysis of Beckman pUC18 control plasmid with the -47 primer on each sequencing plate. Validation of DNA sequencing using the CEQ8000 system is described in detail in [16].

5 Homogeneity

5.1 Planning of homogeneity study

It has been shown previously that the repeatability of the procedure for quantification of gDNA by PicoGreen® can be performed with a CV = 2.4 %, for an analyte mass concentration of 50 ng/mL [14]. The targeted maximum relative contribution of inhomogeneity to the combined standard uncertainty has been set to 6 %. The total number of vials produced was 718. The total number of vials to be used in the homogeneity study (*N*) was calculated from $\sqrt[3]{718} \approx 9$ [4]. In this study *N* was set to 10. Assuming that the repeatability of the procedure expressed as CV = 2.4 % cannot be decreased, u^*_{bb} (uncertainty component due to the inhomogeneity that can be hidden by the repeatability of the procedure) was calculated for different number of replicate measurements *n* (*N* = 10). For *n* = 2, u^*_{bb} is 1.13 and for *n* = 3, u^*_{bb} is 0.78. Therefore, 2 measurements per sample are enough to detect inhomogeneity above 6 %. Nevertheless, in this study three measurements were used in the homogeneity measurements.

5.2 Evaluation of homogeneity study

A homogeneity study was performed on 10 vials with 3 measurements per vial. ds gDNA was quantified by fluorescence of PicoGreen® using lambda phage DNA as external standard [11]. The results of the homogeneity measurements are summarized in Table 3.

Table 3. Homogeneity of the mass of gDNA [ng] per vial of IRMM-448. Vials were stored at -20 ºC.

	mass gDNA [ng]			
Vial number	Measurement 1	Measurement 2	Measurement 3	Average
29	84.2	79.0	72.2	78.5
94	76.5	68.6	73.0	72.7
149	84.5	71.7	79.6	78.6
225	75.6	71.7	71.7	73.0
354	75.1	71.7	81.6	76.1
412	73.5	71.2	70.6	71.8
431	70.2	74.5	75.7	73.5
516	83.6	72.9	66.9	74.5
578	82.6	69.7	75.8	76.0
649	72.9	76.6	73.5	74.4

The average of the study was $\bar{y}$ = 74.9 ng. No outliers were detected (Grubbs test for outliers over sample means), therefore the complete set of data was used. Analysis of the data by ANOVA is summarized in Table 4 (using MS Excel™ single factor ANOVA).

Table 4. Analysis of data from Table 3 using MS Excel™ single factor ANOVA.

	SS	ν	MS
among groups	150.1	9	16.7
within groups	503.6	20	25.2
Total	653.8	29	

From Table 4 the repeatability of the procedure and u^*_{bb} were calculated as 6.7 % and 1.6 %, respectively, according to the procedures described in references [6 - 8]. s_{bb} can not be calculated because $MS_{among} < MS_{within}$. The inhomogeneity is too small to be detected by the study. Based on the repeatability of the procedure and the set-up of the study, an inhomogeneity of maximum 1.6 % could be hidden (u^*_{bb}). Therefore, the uncertainty contribution resulting from the homogeneity assessment of the filling and production process is 1.6 %.

A plot of the mass of gDNA in ng per vial against sample number does not reveal a trend in the filling sequence. The slope of the regression line was statistically insignificant (*t*-test, data not shown).

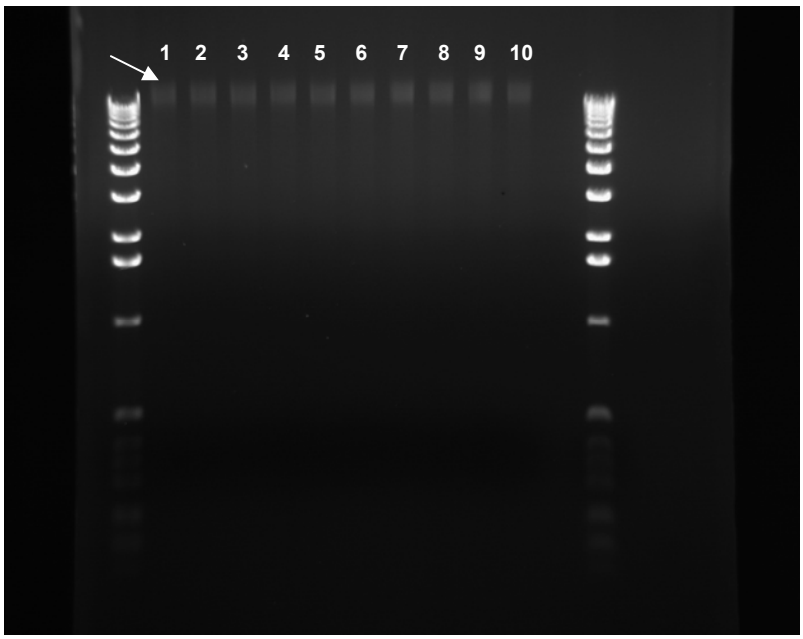


Figure 1. Agarose gel (mass fraction = 0.8 %) electrophoresis of gDNA (arrow) from 10 vials used in homogeneity measurements of IRMM-448. Lane 1-10 corresponds to the series of sample numbers in Table 3. gDNA was dissolved in 50 µL TE (pH 8.0); 10 µL of each sample was mixed with 1.0 µL BlueJuice™ Gel Loading Buffer (Invitrogen), 10 µL of this mixture was loaded per lane. Marker: 1 kb Plus Ready-Load DNA (Invitrogen).

All samples used in the homogeneity study were analysed by agarose gel electrophoresis of gDNA (Fig. 1). A single band of gDNA is observed in each of lanes 1 to 10, confirming the presence and intactness of gDNA in each vial. From each vial it was possible to amplify the 793 bp amplicon of the *C. jejuni ceu*E gene equally well (data not shown).

6 Stability

6.1 Planning

The proper conditions for transport of the material to the customer were determined from a short-term stability study of samples stored at 4 ºC, 18 ºC and 60 ºC with time points of 0, 1, 2 and 4 weeks (reference samples at -20 ºC). At each time/ temperature combination three different vials were tested. The mass of gDNA in ng per vial was determined by fluorescence of PicoGreen® using lambda phage DNA as external standard [11].

6.2 Short-term stability

The data from the short-term stability studies are summarized in Table 5, Fig. 2, 3 and 4. One spurious result was visible. Sample no 15 in Fig. 4 gave no amplification of the *ceu*E gene. This was most likely due to a lab error, because a repeat of the measurement gave a positive result (data not shown). The average mass of gDNA in μg per vial of the t = 0 vials was $\bar{y}$ = 67.1 ng.

At 60 °C the material was unstable and degraded after 4 weeks ($|b|/u_b > t_{0.05,\ 10}$). Visual inspection of gDNA samples incubated at 60 °C by agarose gel electrophoresis also showed that the material was degraded (Fig 2, lanes 22 - 30). From each vial, including vials incubated at 60 °C, it was possible to amplify the *ceu*E gene by qualitative PCR equally well (Fig. 4).

For the three temperatures, the slope of the regression line and its uncertainty were calculated and tested for statistical significance (Table 6). The slope of the regression line at 4 and 18 °C was statistically and technically insignificant ($|b|/u_b < t_{\alpha,\nu}$). Furthermore, no signs of degradation were observed upon inspection by agarose gel electrophoresis of gDNA samples incubated at 4 or 18 °C (Fig. 2).

After 4 weeks storage of gDNA at 60 ºC the PCR product of the *ceu*E amplicon was still detected (Fig. 4). Similar results were obtained for all time-points (Fig. 2 and 4). Therefore, the material can be used as template in PCR reactions even if stored under sub-optimal conditions.

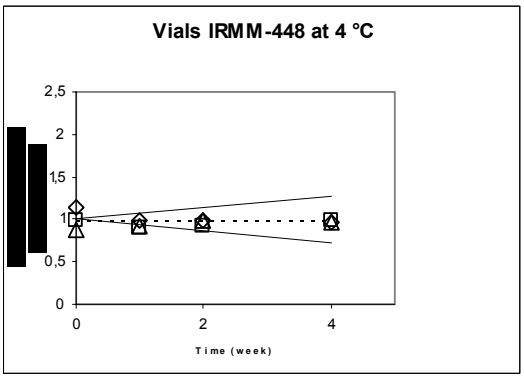
In conclusion, the material shall be dispatched under cooled conditions resulting in negligible uncertainty due to degradation.

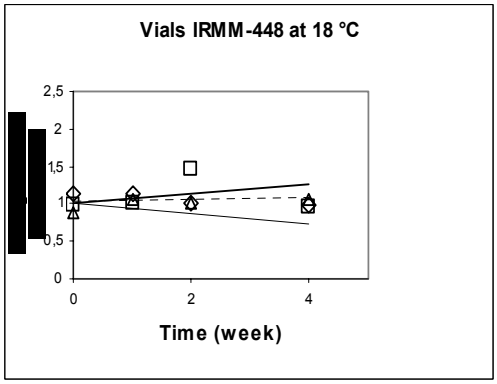
Table 5. Short-term stability of IRMM-448 stored at three different temperatures. At each time/temperature combination three different vials were tested (mass ds gDNA per vial in ng). Each data point is the average of three measurements (*n* = 3).

		mass gDNA [ng]			
	Temperature [°C]	*t* = 0 [week]	*t* = 1 [week]	*t* = 2 [week]	*t* = 4 [week]
Result 1	4	76,1	65,8	65,9	65,1
Result 2	4	65,9	60,9	62,0	66,6
Result 3	4	59,3	62,2	66,7	65,1
Average		67,1	62,9	64,9	65,6
Result 1	18	76,1	76,8	68,5	65,6
Result 2	18	65,9	68,3	98,2	65,0
Result 3	18	59,3	71,2	68,2	70,4
Average		67,1	72,1	78,3	67,0
Result 1	60	76,1	6,7	20,7	12,5
Result 2	60	65,9	8,7	25,4	11,0
Result 3	60	59,3	12,4	21,7	11,8
Average		67,1	9,3	22,6	11,8

Figure 2. Agarose gel (mass fraction = 0.8 %) electrophoresis of gDNA from IRMM-448 stored at different temperatures. Samples were used for stability measurements (cf. Table 5). gDNA was dissolved in 50 µL TE (pH 8.0); 15.0 µL of each sample was mixed with 1.5 µL BlueJuice™ Gel Loading Buffer (Invitrogen), and 15 µL was loaded per lane. Marker: 1 kb Plus Ready-Load DNA (Invitrogen).

Lane	**1, 2, 3:**	**0 week 4 °C**	**lane**	**16, 17, 18:**	**2 weeks 18 °C**
Lane	**4, 5, 6:**	**1 week 4 °C**	**lane**	**19, 20, 21:**	**4 weeks 18 °C**
Lane	**7, 8, 9:**	**2 weeks 4 °C**	**lane**	**22, 23, 24:**	**1 week 60 °C**
Lane	**10, 11, 12:**	**4 weeks 4 °C**	**lane**	**25, 26, 27:**	**2 weeks 60 °C**
Lane	**13, 14, 15:**	**1 week 18 °C**	**lane**	**28, 29, 30:**	**4 weeks 60 °C**
			lane	**31:**	**1 week 18 °C**





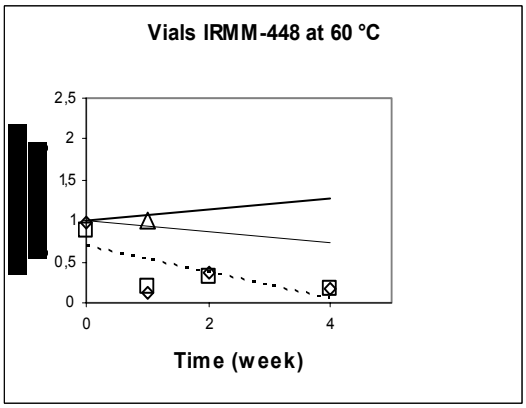
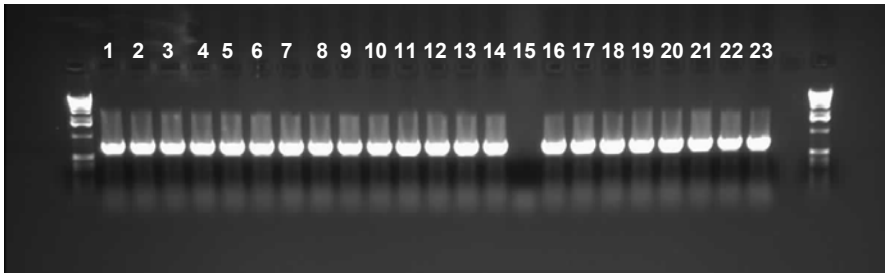


Figure 3. Stability measurements for IRMM-448. The mass of gDNA per vial was quantified by fluorescence of PicoGreen® using lambda phage DNA as external standard [8]. Values were normalised to 1.0 for *t* = 0. The dashed lines present the calculated regression lines from Table 6. The solid lines indicate the theoretical expected range of values applying a calculated u_{lts} = 27.2 %. At each time/temperature combination three different vials were tested: ◊, first measurement result; □ , second measurement result ; Δ, third measurement result.

Table 6. Calculation of regression lines, their uncertainty and statistical significance. All data were normalised by setting the average value $\bar{y}$ at *t* = 0 to 1.0.

Temperature [°C]	slope *b* [week^{-1}]	intercept *a*	relative u_b [week^{-1}]	$\lvert b \rvert / u_b$	$t_{\alpha,\nu}$
4	-0.001	0.973	0.013	0.077	2.228 ($t_{0.975,\ 10}$)
18	-0.002	1.063	0.030	0.067	2.228 ($t_{0.95,\ 10}$)
60	-0.157	0.687	0.057	2.754	2.228 ($t_{0.95,\ 10}$)



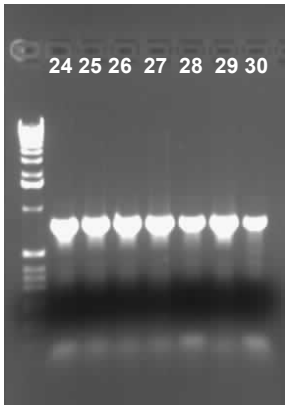


Figure 4. Agarose gel (mass fraction = 1.2 %) electrophoresis of the 793 bp PCR product from *C. jejuni ceu*E gene. The samples used for stability measurements and stored at different temperatures as indicated (cf. Table 5) were used as templates in PCR reactions using primers JEJ1 and JEJ2. 25 µL of each PCR reaction was mixed with 2.5 µL BlueJuice™ Gel Loading Buffer (Invitrogen), and 15 µL was loaded per lane.

lane	**1, 2, 3:**	**0 week 4 °C**	**lane**	**16, 17, 18:**	**2 weeks 18 °C**
lane	**4, 5, 6:**	**1 week 4 °C**	**lane**	**19, 20, 21:**	**4 weeks 18 °C**
lane	**7, 8, 9:**	**2 weeks 4 °C**	**lane**	**22, 23, 24:**	**1 week 60 °C**
lane	**10, 11, 12:**	**4 weeks 4 °C**	**lane**	**25, 26, 27:**	**2 weeks 60 °C**
lane	**13, 14, 15:**	**1 week 18 °C**	**lane**	**28, 29, 30:**	**4 weeks 60 °C**

6.3 Long-term stability

No long-term stability study was performed. As an estimation of u_{lts} a value of u_{lts} = 27.2 % (relative u_{lts} for 5 years) was used. This value was obtained in the long-term stability study of a similar reference material, IRMM-449, gDNA of *Escherichia coli* O157 [3]. The use of this value of u_{lts} is justified because only the DNA sequence and the mass per vial of IRMM-448 are different as compared to IRMM-449. This does not change the "nature" of the material, i.e. both CRMs are composed of gDNA and consequently this does not influence long-term stability. When new data will come available from Post Certification Monitoring the u_{lts} will be recalculated and a new certificate could be issued.

7 Batch characterisation

The data from the homogeneity study and t = 0 vials in the short-term stability study were used for batch characterization.

8 Additional characterisation

8.1 DNA sequence

Plasmid DNA containing the 793 bp amplicon of the *ceu*E gene was purified and used for dye terminator cycle sequencing using sequencing primer M13-Forward (Invitrogen, US). The analysed DNA sequence of 242 bp showed 94 to 99 % homology with the reported DNA sequence of the *ceu*E gene of different strains of *C. jejuni* confirming the authenticity of the produced gDNA (Fig. 5). Bio-informatic tools using Nucleotide-nucleotide Blast functions reveal differences between organisms and strains by identifying nucleotide changes, whereas confirming strain or protein- and nucleotide sequence identity.

The characterisation of identity based on the sequence information is in agreement with additional identity information (biochemical-, morphological- and microbiological information) from the certificate of analysis of NCTC for *C. jejuni* (NCTC 11351).

```
gi|757794|emb|X82427.1|CJBPGENE  C.jejuni binding protein gene
Length=1581

 Score =  410 bits (207),  Expect = 2e-111
 Identities = 242/244 (99%), Gaps = 2/244 (0%)
 Strand=Plus/Minus

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Sbjct  1027  TGATCTTTTTGTTTTGTGCTGCTTTGGTTTTAGCGACAAGTGCATTATCGAGTATGCCTT  968

Query  142   GAGCGCGTTCTTTGTTGCCTACAATGATATTTCTATCAATAACAAAAATATAATCAGGAt  201
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Sbjct  788   AAGG  785
```

Figure 5. Comparison of the homology of the DNA sequence of the *ceu*E amplicon obtained from IRMM-448 (Query) with the reported DNA sequence of the *ceu*E gene (Genebank accession no. AL111168) using Nucleotide-nucleotide BLAST (blastn) on www.ncbi.nlm.nih.gov.

8.2 Specificity of ceuE primer set

PCR reactions were performed using IRMM-448 gDNA as template DNA with PCR primer sets for the identification of *Listeria monocytogenes* 4B (NCTC 1194) or *Escherichia coli O157* EDL933 (*cf.* Table 2). No PCR products were observed from these reactions (data not shown), confirming the specificity of the primer set used for the detection of the *ceu*E gene.

9 Metrological traceability

The traceability of results obtained by gravimetry, volumetry, thermometry and spectrophotometry were demonstrated and documented.

The indicative value (mass of gDNA per vial) is traceable to the SI via lambda DNA standard solutions, prepared according to the procedure described in reference [11].

The results for the certification of IRMM-448 obtained by qualitative PCR detection of the *ceu*E gene are traceable to the procedure described in references [1, 9].

The certified identity of the genomic DNA has been confirmed by dye terminator cycle sequencing of the *ceu*E gene [15].

10 Indicative value and related uncertainty

In order to verify a normal Gaussian distribution, normal probability plots were prepared of the homogeneity data versus their normal quantile values (data not shown). The straight line obtained in this plot was an indication of a normal Gaussian distribution of the data [6]. Therefore, the indicative value for the mass of gDNA per vial is calculated as the unweighted average of the average of the t = 0 vials in the short-term stability study (67.1 ng) and the average from vials of the homogeneity study (74.9 ng). This value is 71.0 ng gDNA per vial.

In Table 7 the uncertainty budget for IRMM-448 is summarized. The relative uncertainty resulting from the homogeneity assessment (u^*_{bb}) is 1.6 %. The relative uncertainty resulting from the stability assessment ($u_{rel,\ lts}$) is 27.2 % [3]. The relative uncertainty from the characterisation, using CV = 4.3 % [14], is estimated as: u_{char} = 4.3 %/√2 = 3.0 %. Using a coverage factor k = 2 (as defined in reference [8]) the certified, expanded uncertainty is calculated as:

$U_{CRM} = 2*\sqrt{(0.016^2 + 0.272^2 + 0.030^2)} \times 100 = 54.8$ %.

This corresponds to 38.9 ng.

Table 7. Uncertainty budget IRMM-448. Coverage factor *k* = 2 [8].

Component (assessment)	Relative standard uncertainty [%]
u^*_{bb} (homogeneity)	1.6
$u_{rel,\ lts}$ (long term stability)	27.2
u_{char} (batch characterisation)	3.0

11 Instructions for use

11.1 Dispatch

Dispatch to the customer can be done under cooled conditions. Upon receipt by the customer, the material should be kept at -20 °C until the expiry date.

11.2 Instructions for use

This CRM is intended to be used as positive control material for the identification and detection of *Campylobacter jejuni* by diagnostic PCR.

Vials have to be kept at -20 °C until use. Dried gDNA should be reconstituted by the following procedure:

1. Centrifuge vial for 1 min at 5 900 × *g* to ensure that the lyophilised material is at the bottom of the vial.
2. Add 50 µL DNase free, sterile TE buffer (pH 8.0) and gently swirl the vial.
3. Shortly centrifuge vial during 4 - 10 s.
4. Use 1 µL of reconstituted gDNA per 25 µL total volume in the PCR-reaction (depending on application and amplicon).
5. After reconstitution, gDNA can be kept at 4 °C for four weeks or at -20 °C until the expiry date.

The procedures used in this report are described in more detail elsewhere [1, 2 and 9].

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Abstract

This report describes the production and certification of a reference material of purified genomic DNA (gDNA) of *Campylobacter jejuni* (NCTC 11351). This CRM (IRMM-448) supports the harmonisation and validation of PCR procedures by their use as taxonomic controls in PCR reactions. The homogeneity and stability (at three different temperatures for up to 4 weeks) of the batch was assessed by monitoring the amplification of the *ceu*E gene by PCR, by agarose gel electrophoresis of gDNA and determination of the mass of gDNA per vial. The material is stable at 4 and 18 °C. The identity of the gDNA was confirmed by DNA sequence analysis of the *ceu*E gene. Each vial contains a certified mass of 71 ng freeze-dried gDNA, with an expanded uncertainty (U_{CRM}) of 39 ng (k = 2).

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