Final report of CCQM-K86.c Relative quantification of genomic DNA fragments extracted from a biological tissue

Study organizers:
Zoltan Mester and Philippe Corbisier

Authors:
Zoltan Mester[1], Philippe Corbisier[2], Stephen L. R. Ellison[6]

Maria Roxana Beaz Hidalgo[2], Yunhua Gao[3], Chunyan Niu[3], Vincent Tang[4], Foo-wing Lee[4], Melina Pérez-Urquiza[5], Angel Ramirez Suárez[5], Malcolm Burns[6], Mojca Milavec[7], Kanjana Wiangnon[8], Kate R. Griffiths[9], Jacob L.H. McLaughlin[9], Sachie Shibayama[10], Akiko Takatsu[10], Muslum Akgoz[11], Maxim Vonsky[12], Andrei Runov[12], John Emerson Leguizamon Guerrero[13].

Affiliations
[1]National Research Council Canada
[2]Joint Research Centre, Geel, EU
[3]National Institute of Metrology P. R. China, Beijing, China
[4]Government Laboratory Hong Kong, Kowloon, Hong Kong
[5]Centro Nacional de Metrología, Querétaro, México
[6]LGC, Teddington, United Kingdom
[7]National Institute of Biology, Ljubljana, Slovenia
[8]National Institute of Metrology Thailand, Pathumthani, Thailand
[9]National Measurement Institute Australia, Lindfield, Australia
[10]National Metrology Institute of Japan, AIST, Japan
[11]TUBITAK/UME (National Metrology Institute), Gebze, Kocaeli, Turkey
[12] D.I.Mendeleev Institute for Metrology, Russia
[13]Instituto Nacional de Metrologia de Colombia

Abstract

Key comparison CCQM-K86.c was performed to demonstrate the capacity of National Metrology Institutes (NMIs) and Designated Institutes (DIs) in the determination of the relative quantity of two specific genomic DNA fragments present in a canola powder. The study provides direct support for the following measurement claim: "**Quantification of the ratio of the number of copies of specified intact sequence fragments of a length up to 150 nucleotides following extraction from an unprocessed, high fat/oil ground seed matrix, with a copy number ratio from 0.001 to 1**".

The study was carried out under the auspices of the Nucleic Acids Working Group (NAWG) of the Consultative Committee for Amount of Substance: Metrology in Chemistry and Biology (CCQM) and was jointly coordinated by the National Research Council of Canada (NRC) and the EU Joint Research Centre, Geel (JRC). The following laboratories (in alphabetical order) submitted measurement results in this key comparison study: Centro Nacional de Metrología, Mexico ("CENAM"); D.I. Mendeleyev Institute of Metrology, Russia ("VNIIM"); EU Joint Research Centre, Geel (JRC); Hong Kong Government Laboratory ("GLHK"); Instituto Nacional de Metrología de Colombia ("INM"); LGC (United Kingdom); National Institute of Biology, Slovenia ("NIB"); National Institute of Metrology, P.R. of China ("NIM China"/"NIMC" [figures]); National Institute of Metrology, Thailand (NIMT); National Measurement Institute, Australia ("NMIA"); National Metrology Institute of Japan, AIST, Japan ("NMIJ"); National Metrology Institute of Turkey ("TÜBITAK").

1 Introduction

Genetically modified (GM) crops are now ubiquitous in the global feed, fiber, food, and biofuel applications. However, the regulatory framework around the approval and use of GM traits and crops differs significantly in various jurisdictions. Consequently, the accurate quantification of GM content in such products represents a technical international trade issue.

The quantification of the relative abundance of two DNA fragments, one being present at a ratio between 1/1000 and 1/10, in a difficult matrix such as plant material, has been

demonstrated in previous Key Comparisons (CCQM-K86 [1] and CCQM-K86.b [2]).
The matrix in CCQM-K86 was a maize seed powder, whereas in CCQM-K86.b it was a rice seed powder. Both matrixes are rich (between 75 and 80 g/100g) in polymeric carbohydrate (amylose and amylopectin) and poor in fat (<5 g/100g) requiring adapted DNA extraction method to remove substances that can hinder the polymerase activity.
In this study, the NAWG decided to challenge laboratories with a plant material with elevated oil/fat content, such as rapeseed. As the rapeseed is genetically engineered, validated DNA extraction and qPCR quantification methods are available.
The quantification was performed by quantitative digital Polymerase Chain Reaction (dPCR). The methodology requires extraction and purification of genomic DNA and accurate detection and quantification of the relative amount of two defined DNA sequences in the extracted genomic DNA. It was agreed to organize two studies in parallel: a key comparison, CCQM-K86.c and a concurrently run pilot study, CCQM-P113.4, based on materials provided by NRC and JRC Geel.

2 Measurement Claim

The measurement claim for CCQM-K86.c is " Quantification of the ratio of the number of copies of specified intact sequence fragments of a length up to 150 nucleotides following extraction from an unprocessed, high fat/oil ground seed matrix, with a copy number ratio from 0.001 to 1".
The lower limit for fragment length depends on the particular primer set. A capability for measurement of a copy number ratio of 0.001 indicates a capability to measure a copy number ratio of 1000 (1/0.001).
The study demonstrates the participants' competence to extract DNA from an oil rich matrix and to perform measurements on the extracted DNA using dPCR.

3 Participation in CCQM-K86.c

The 12 national Measurement Institutes and Designated Institutes ("participants") that participated in CCQM-K86.c are listed in Table 1. A protocol (Appendix A) was sent to all participants prior to sample distribution. The protocol provided information concerning the storage and analysis of the samples. Participants were free to use a method of their choice

for the determination of the copy number ratio. They were asked to report results as copy number ratio, expressed as a percentage, on the two unknown samples as received.

Table 1. CCQM-K86.c participants.

Institute / Organisation	Country	Contact
JRC Geel	EU	P. Corbisier
NIMT	Thailand	K Wiangnon
NMIA	Australia	K. Griffiths
TUBITAK	Turkey	M. Akgoz
NMIJ	Japan	S. Shibayama
VNIIM	Russia	M. Vonsky
LGC	UK	M. Burns
INM	Colombia	J. E. Leguizamon Guerrero
NIB	Slovenia	M. Milavec
CENAM	Mexico	M. Pérez-Urquiza
GLHK	Hong Kong	V. Tang
NIM China	China	L. Dong

4 Study Materials

4.1 Test samples

The participating laboratories were provided with two unknown samples:

Sample T1: One rapeseed powder containing a defined mass fraction of genetically modified (GM) DP-Ø73496-4 rapeseed (*Brassica napus L.*). The samples were produced under the responsibility of the JRC and were prepared by mixing of ground full-fat non-GM rapeseed and ground full-fat DP-Ø73496-4 GM rapeseed.

Sample T2: One rapeseed powder containing a defined mass fraction of genetically modified (GM) GT73/RT73 rapeseed (*Brassica napus L.*). The samples were produced under the responsibility of NRC and were prepared by mixing of commercially processed non-GM rapeseed powder and ground GT73/RT73 rapeseed. **Note**: Commercial processing results in a reduction in oil content.

4.2 Homogeneity of the test samples

Homogeneity assessment of the mass fraction in samples T1 and T2 was performed by JRC and NRC respectively, and the uncertainty related to the homogeneity was provided to participants as a relative standard uncertainty. For T1, the relative standard uncertainty related to an estimate of the maximum between-unit heterogeneity that could be hidden by the intermediate precision of the qPCR method was 5.2 % [3]. For T2, the relative standard uncertainty related to the homogeneity was 5.5 % [4]. The sample intake used for determining the homogeneity was 200 mg for both samples T1 and T2.

4.3 Stability of the test samples

For sample T1 JRC performed a short-term stability study using an isochronous design. In this approach, samples were stored for a certain time at different temperature conditions. After the planned exposure times, the samples were moved to conditions where further degradation can be assumed to be negligible (reference conditions). At the end of the isochronous storage, the samples were analysed simultaneously under intermediate precision conditions. The study concluded that the samples could be dispatched without further precautions under ambient conditions [3].

The stability of sample T1 had also been monitored since production and the material was found stable at 4°C over a period of 6 years [3]. Sample T2 was found to be stable for over 10 years [4].

5 Methods and instrumentation

Sample T1 was dispatched from JRC; sample T2 from NRC Canada. General advice to participants included:

a) Participants were requested to use their preferred methodology for the extraction of genomic DNA from samples T1 and T2. Attention was, however, drawn to the extraction method referred to by the European Union reference Laboratory for GM Food and Feed [5].
b) Special care was recommended in preparing sufficiently purified genomic DNA.
c) Dilution of the unknowns was suggested in order to verify the absence of PCR inhibitors in the extracted DNA.
d) Digital PCR is the preferred method to quantify DNA sequences in the absence of calibrant solutions but other technologies could also be used.
e) Participants were permitted free choice of digital PCR platform and chemistry.

In addition, a detailed study protocol was provided, giving information on study timescale, sample preparation, suggested methodology, probe and primer sequences, and reporting requirements was provided; the study protocol is included as Appendix A of this report

6 Measurands and reporting requirements

Participants were required to report:

- The absolute copy number of both sequence targets for samples T1 and T2 per µL DNA in the PCR MasterMix;
- The ratio of both sequence targets expressed in percent for samples T1 and T2 as well as the uncertainty;
- An outline of the methodology, a measurement equation and a breakdown of the uncertainty estimation submitted.

Table 2 summarizes the extraction methods, instrumentation and the chemistries, used by the participants.

Table 2. Analytical methods and instrumental techniques used by participants in CCQM-CCQM-K86.c.[Note 1]

Institute	Extraction method (sample intake)	Instrument	PCR Reagents	Primers and probes used for T1	Primers and probes used for T2
JRC Geel (IRMM)	CTAB-Tip20 (200 mg)	BioRad QX-100 + C1000 Touch™ Thermo cyclerThermal Cycler	ddPCR™ Supermix	As defined in Appendix A DP-073496-4 fatA(A)	As defined in Appendix A RT73 fatA
NIMT	EURL GMFF protocol (200 mg)	Bio-Rad QX200	-	As defined in Appendix A DP-073496-4 fatA(A)	As defined in Appendix A RT73 fatA
NMIA	in-house DNA isolation with paramagnetic beads	Bio-Rad QX100	-	As defined in Appendix A DP-073496-4 fatA(A)	As defined in Appendix A RT73 fatA
TUBITAK	CTAB	QuantSudio3D Digital PCR 20K	Master Mix (Thermo Fisher Scientific)	As defined in Appendix A DP-073496-4 fatA(A)	As defined in Appendix A RT73 fatA(A)
NMIJ	GM quicker 2 (NipponGene, Japan)	QuantStudio 3D Digital PCR system	Master Mix v2 (Thermo Fisher Science Inc.)	As defined in Appendix A DP-073496-4 fatA(A)	As defined in Appendix A RT73 fatA
VNIIM	EURL GMFF protocol (200 mg).	BioRad QX-200 + C1000 Touch Thermal Cycler	2x ddPCR Super Mix for Probes	As defined in Appendix A DP-073496-4 fatA(A) + locked dGTP	As defined in Appendix A RT73 fatA(A)
LGC	-	Bio-Rad QX200	-	As defined in Appendix A DP-073496-4 fatA(A)	As defined in Appendix A RT73 fatA
INM	EURL GMFF protocol (200 mg)	BioRad QX200	ddPCR SuperMix (no UTP)	As defined in Appendix A DP-073496-4 fatA(A)	As defined in Appendix A RT73 fatA(A)

Institute	Extraction method (sample intake)	Instrument	PCR Reagents	Primers and probes used for T1	Primers and probes used for T2
NIB	CTAB (02G-Pos06): 100 µl H2O	BioRad QX100 (20µl reactions)	-	As defined in Appendix A DP-073496-4 fatA(A)	As defined in Appendix A RT73 fatA(A)
CENAM	Fast ID method (300 mg)	Bio-Rad QX200	-	As defined in Appendix A DP-073496-4 fatA(A)	As defined in Appendix A RT73 fatA
GLHK	DNeasy® mericon® Food Kit	Bio-Rad QX200 + C1000 Touch™ Thermal Cycler	ddPCR™ Supermix	As defined in Appendix A DP-073496-4 fatA(A)	As defined in Appendix A RT73 fatA
NIM China	Wizard® magnetic purification/ DNAsecure plant kit/ DNeasy® plant mini kit	Biomark (T1) Biomark, QX200 (T1, T2)	-	As defined in Appendix A DP-073496-4 fatA(A)	As defined in Appendix A RT73 fatA(A)

Note 1: "-" indicates no additional information available

7 Results

7.1 CCQM-K86.c participants' results

Measured replicate copy number concentrations of both sequence targets for samples T1 and T2 per µL DNA as well as the final dilution of the DNA in the PCR assays are presented in Tables 3 and 4, respectively. All participants quantified the respective targets by simplex PCR, except INM which analysed samples T1 and T2 in duplex PCR.

The number of copies of the DNA sequence targets that are assayed in a given PCR reaction depends on both the concentration of the DNA solution added to the dPCR and the volume of the DNA solution added to the dPCR. Tables 3 and 4 show that the number of copies of the DNA sequence targets were assayed at different concentrations by different participants. The less abundant target (for DP-Ø73496-4) present in T1 had to be tested using an undiluted DNA extract or (for higher copy number per partition) could be concentrated as did NMIJ, whereas the DNA extracts used for the endogenous targets (FATA(A)) assay had to be diluted first. The same strategy could be applied for the T2 sample even if the transgenic target was about 50 times more abundant than the DP-Ø73496-4 target. Not all participants optimised the concentration of the DNA sample to quantity the endogenous targets. Some participants quantified the DP-Ø73496-4 target at a very low copy number (probably very close to the LOQ of their method).

Table 3. CCQM-K86.c participants' measurement results for sample T1.

NMI/DI	DP-Ø73496-4 target		FatA(A) target	
	copy number concentration (cp/µL)	final dilution	copy number concentration (cp/µL)	final dilution
IRMM[1]	193 157 130 179	2.857 2.857 2.857 2.857	170047 180547 163154 165482	77.139 77.139 77.139 77.139
NIMT	178.7 151.8 146.6 169.5 159.0	10 10 10 10 10	183032 154799 142509 150043 159814	1000 1000 1000 1000 1000
NMIA[1,2]	59.2 64.2 40.1 18.3 25.7 25.4	2.625 2.596 2.595 2.618 2.656 2.611	48900 55400 43200 18200 26000 25700	53.592 55.020 54.863 51.576 51.911 51.176
TUBITAK	140 143	2.415 2.427	132415 135561	65.476 67.063
NMIJ	1069 1114 1112	5.069 5.061 5.190	980367 1030279 990469	476.948 504.091 477.024
VNIIM	8.6 20.7 14.1	4 4 4	8977 17650 13050	40 40 40
LGC	119 131 189 123	39.822 40.687 41.447 40.576	100289 119675 126386 117710	39.822 40.687 41.447 40.576
INM[1]	159 169 134 159 217 176 204 182 128	180.6 188.9 190.6 197.2 222.2 183.3 216.7 215.3 161.1	153867 166537 126138 143595 147219 115512 170169 161959 135605	180.6 188.9 190.6 197.2 222.2 183.3 216.7 215.3 161.1
NIB[1]	17 12 9	5 5 5	9489 8709 9933	5 5 5
CENAM	65	5	45400	20
GLHK	5.99 5.38 6.18 6.40	3.991 2.011 4.105 2.009	4122 4412 4275 4442	3.991 4.040 4.105 8.049
NIM China[1,2]	32.57 33.99 40.48 17.86 18.13	1 1 1 1 1	22639 22743 26122 10519 11393	104.1 104.1 104.1 15.03 15.03

n.p.: not provided; [1]: reported by individual extraction; [2]: tested at two concentration levels.

Table 4. CCQM-K86.c participants' measurement results for sample T2.

NMI/DI	RT73 target		FatA target	
	copy number concentration (cp/µL)	final dilution	copy number concentration (cp/µL)	final dilution
IRMM[1,2]	5966	2.857	296396	171.429
	15149	7.143	779091	356.571
	14590	7.143	753974	377.143
	15632	5.714	745648	356.571
NIMT	19337	200	905596	2000
	19791	200	1204671	2000
	21762	200	1163704	2000
	18423	200	970579	2000
	36267	200	1676120	2000
	17924	200	998353	2000
	18747	200	981621	2000
NMIA[1,2]	3620	2.588	126000	82.368
	5410	2.608	166000	77.001
	4640	2.597	148000	77.660
	15200	30.772	471000	324.016
	10800	26.695	364000	292.133
	10500	26.309	367000	280.047
TUBITAK[1,3]	7996	25.250	162901	100.806
	19640	54.644	354580	228.035
NMIJ	22350	104.191	833110	503.572
	24067	105.512	784616	529.133
	21961	105.408	743460	523.318
VNIIM[3]	1556	8	28320	80
	1460	8	25480	80
	1616	8	28900	80
	1872	8	35253	80
	1788	8	32547	80
LGC	14352	242.408	575038	242.408
	14813	247.037	571363	247.037
	17281	252.480	603463	252.480
	15902	252.878	611920	252.878
INM[1]	12732	376.7	417283	376.7
	15359	473.3	523757	473.3
	10163	305.0	326292	305.0
	14781	276.7	500522	276.7
	18753	476.7	680000	476.7
	13829	340.0	510607	340.0
	21334	540.0	727771	540.0
	23348	446.7	814949	446.7
	37688	683.3	1243197	683.3
NIB[3]	2767	5	60892	50
	2949	5	65081	50
	2460	5	51220	50
CENAM	2716	20	93708	40
GLHK	145	7.98	4273	16.028
	147	4.027	4443	8.094
	139	2.010	4023	8.087
NIM China[2,3]	31.23	18	706.33	18
	6.67	12	144.79	12
	7.88	18	172.71	18
	8.04	18	168.00	18
	15.95	15	482.92	15
	41.96	30	1285.03	30
	57.14	10	1353.13	10
	54.83	10	1312.25	10
	79.31	10	2047.50	10

n.p.: not provided; [1]: reported by individual extraction; [2]: tested at two concentration levels; [3]: reported as Fat(A) but tested with FatA(A).

The reported ratio of both sequence targets expressed in percent for samples T1 and T2 as well as the uncertainty as reported by the participants are presented in Tables 5 and 6. The results are shown graphically in Figures 3 and 4. The median of the study results and the values are shown on Figures 1 and 2.

Consistency plots in figure 3 and 4, show significant pairwise differences in sample T1 only for the NIM China results, while for sample T2, multiple results at both low and high extremes show significant inconsistencies.

Inspection of the methods used by participants for sample T2 showed that the laboratories reporting the four highest results in the key comparison had used the same reference gene for the GM ratio as for sample T1 (see annotation in Figure 2). For sample T2, this is expected to result in overestimation of the required copy number ratio (see Discussion, below).

Table 5. CCQM-K86.c participants' measurement results for sample T1.

Participant	Reported results x (cp/cp) (%)	Standard uncertainty u (cp/cp) (%)	Expanded uncertainty U (cp/cp) (%)
IRMM	0.10	0.009	0.03
NIMT	0.102	0.003	0.007
NMIA	0.105	0.007	0.015
TUBITAK	0.105	0.007	0.015
NMIJ	0.110	0.008	0.015
VNIIM	0.110	0.013	0.026
LGC	0.120	0.013	0.04
INM	0.12	0.023	0.046
NIB	0.130	0.048	0.096
CENAM	0.14	0.04102	0.08204
GLHK	0.140	0.023	0.045
NIM China	0.160	0.010	0.020

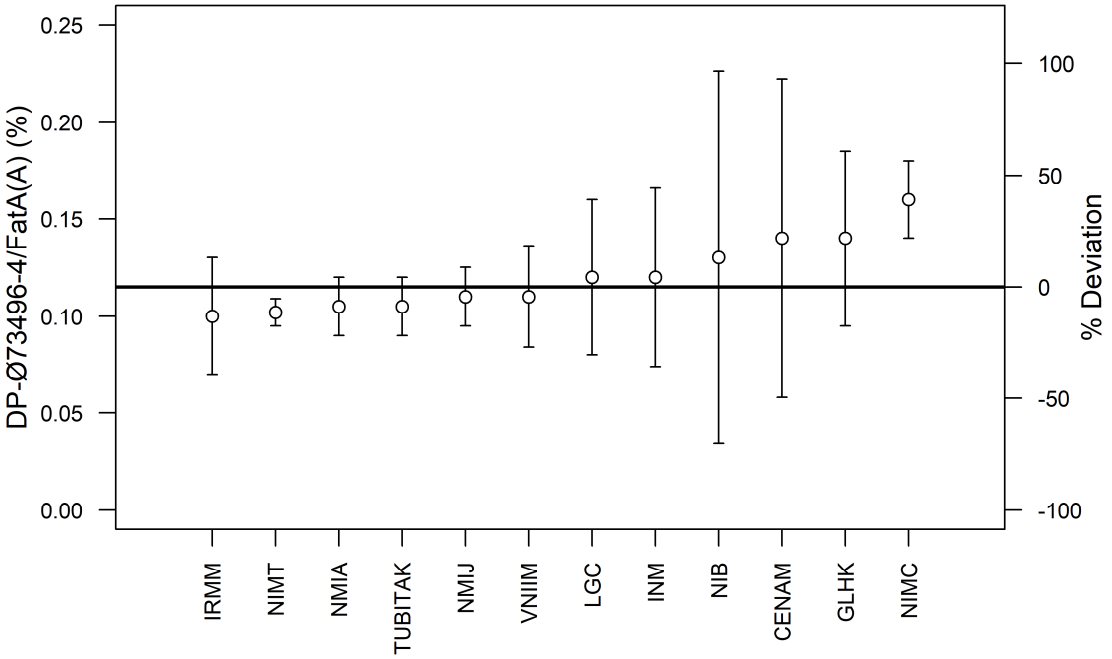

Figure 1. Reported results with expanded uncertainties: Sample T1.

Results are shown as copy number ratio (cp/cp) expressed as a percentage.
The horizontal line shows the median (solid line).

Table 6. CCQM-K86.c participants' measurement results for sample T2.

Participant	Reported results *x* (cp/cp) (%)	Standard uncertainty *u* (cp/cp) (%)	Expanded uncertainty *U* (cp/cp) (
NIMT	1.918	0.077	0.155
IRMM	2.00	0.13208	0.42
LGC	2.6	0.09428	0.300
CENAM	2.9	0.1392	0.2784
NMIJ	2.91	0.185	0.37
INM	2.93	0.17	0.34
NMIA	3.06	0.088	0.21
GLHK	3.39	0.415	0.83
NIM China*	4.1	0.55	1.1
NIB*	4.63	0.19	0.38
TUBITAK*	5.22	0.27876	0.63
VNIIM*	5.52	0.64	1.28

*fatA(A) reference gene

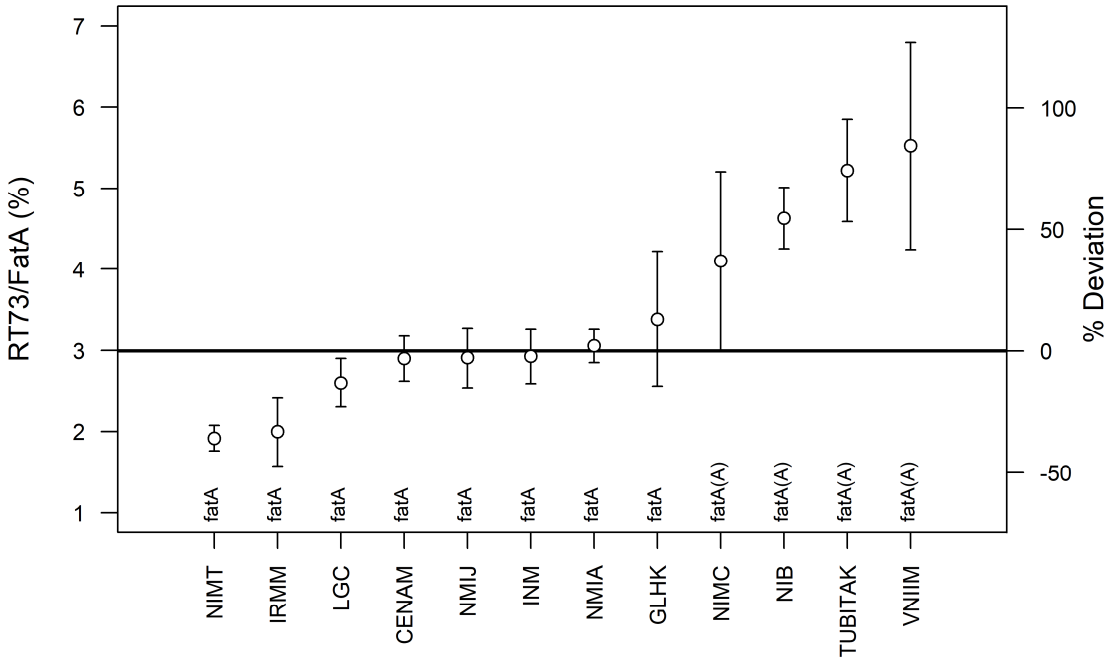

Figure 2. Reported results with expanded uncertainties: Sample T2.

Results are shown as copy number ratio (cp/cp) expressed as a percentage. The horizontal line shows the median (solid line). The reference gene used by each laboratory [fatA vs. fatA(A)] is shown above the *x*-axis.

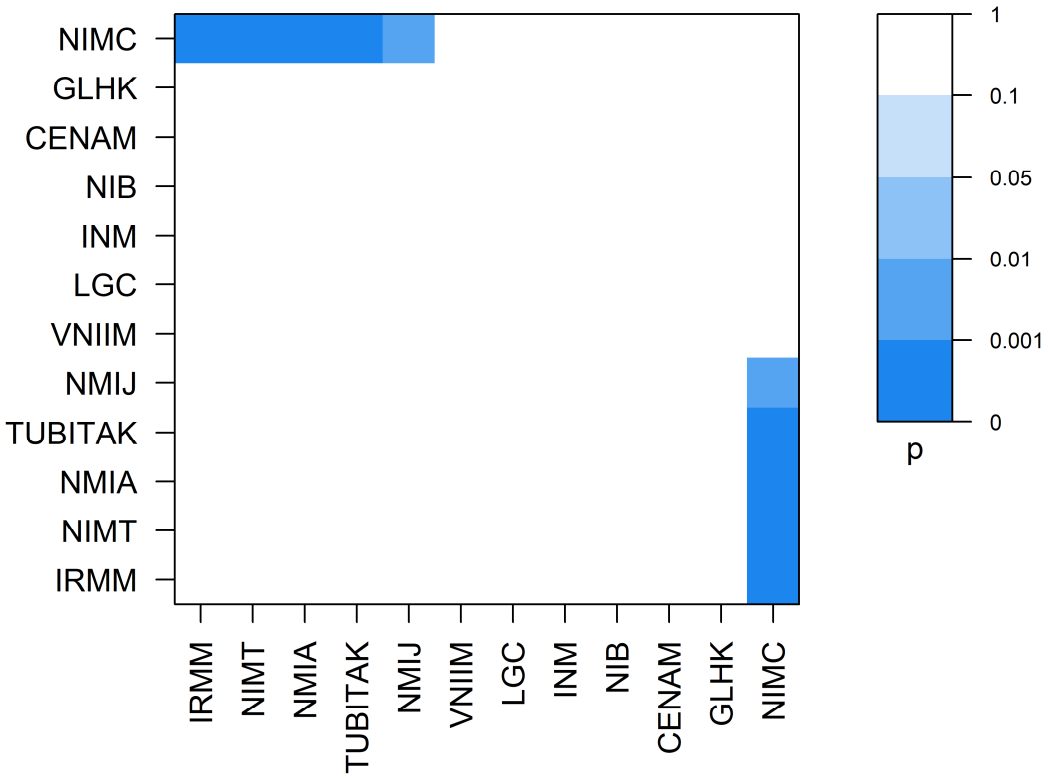

Figure 3. Pairwise consistency plot, Sample T1.

The blue scale shows the *p*-value for a pairwise test of significance of the difference between two laboratories given their reported uncertainty. *p*-values are adjusted for multiple comparisons using Holm's correction [6]

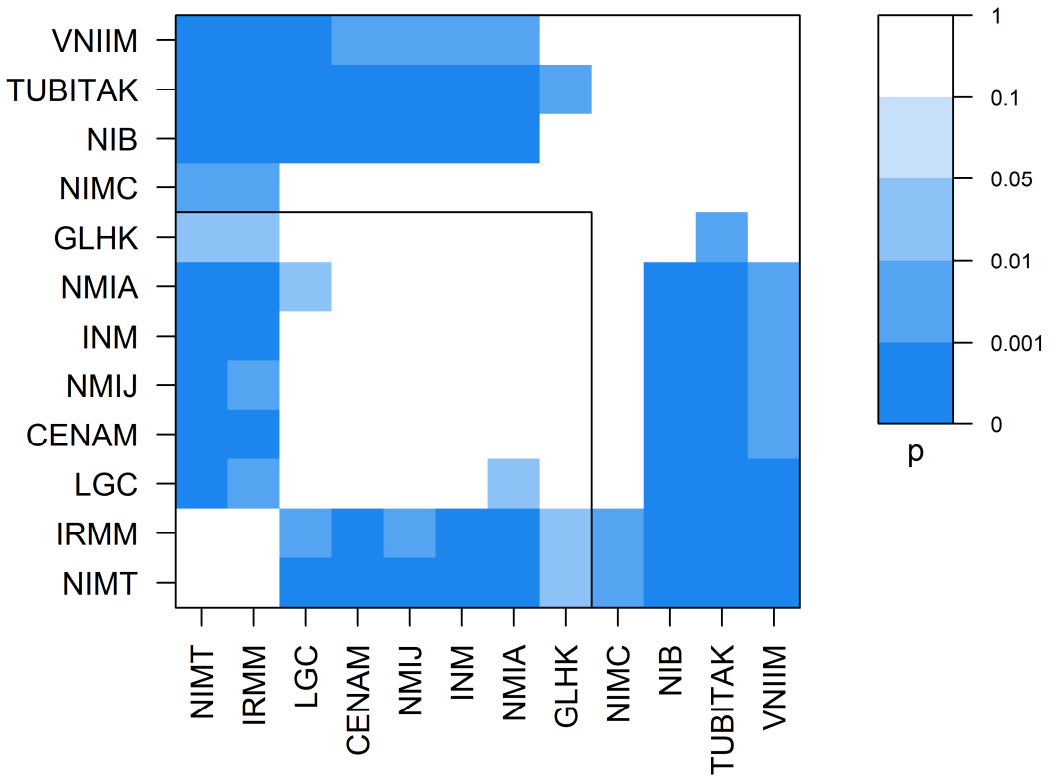

Figure 4.Pairwise consistency plot, Sample T2.

The blue scale shows the *p*-value for a pairwise test of significance of the difference between two laboratories given their reported uncertainty. *p*-values are adjusted for multiple comparisons using Holm's correction [1]. The solid rectangle encloses participants using the recommended reference gene.

7.2 Key comparison reference value (KCRV)

The key comparison reference value (KCRV) was estimated following the CCQM guidance note CCQM13-22 [7]. All CCQM-K86.c participants' results were used to calculate the KCRV for T1; only participants which used the prescribed reference genes were included in the KCRV calculation for T2. NIM China, NIM, TUBITAK and VNIIM data were excluded from KCRV calculation for sample T2 because they used the reference gene fatA(A) instead of the prescribed fatA gene, effectively estimating the value of a different measurand.

Overall the results were remarkably consistent considering the overall complexity of the methodologies especially for sample T1. For sample T2 larger dispersion was observed. The working group concluded that the overall discrepancy would warrant the use of estimators more robust that a simple arithmetic means. Also there has been a significant debate on using robust estimators which are using the reported uncertainties as input variables.

A number of candidate KCRVs are compared in Table 7 and Figure 5. The candidate KCRVs for Sample T1 vary from 0.105 to 0.120 depending on the estimator used, with a standard uncertainty between 0.002 and 0.006. The candidate KCRVs for Sample T2 vary from 2.51 to 2.90 depending on the estimator used, with a standard uncertainty between 0.04 and 0.25. Figures 5a) and 5b) give a graphical representation of the same data.

Noting a level of disagreement around the practices of various laboratories in arriving at their uncertainty estimates, the comparative similarity of results for outlier-resistant estimators, and the evidence of excess dispersion visible in non-zero between-laboratory standard deviations from the REML and DSL estimators, it was decided that a simple median calculation should be used for KCRV assignment.

Table 7. Candidate KCRV estimators

	Estimator	KCRV (cp/cp, %)	*u* (cp/cp, %)	DF	*U* (cp/cp, %)	Remarks
Sample T1	Arithmetic mean	0.1202	0.0054	11	0.0119	Arithmetic mean
	Median/MADe	0.1150	0.0062	11	0.0136	Median/MADe basis
	DerSimonian-Laird	0.1146	0.0053	11	0.0116	Between-lab SD: 0.0132
	REML	0.1153	0.0060	11	0.0132	Between-lab SD: 0.0159
	Weighted mean	0.1074	0.0022	11	0.0048	Weighted mean
	H15	0.1191	0.0068	11	0.0149	Huber (omitting u[i])
	Huber	0.1053	0.0023	11	0.0050	Huber (including u[i])
Sample T2	Arithmetic mean	2.7135	0.1819	7	0.4301	Arithmetic mean
	Median/MADe	2.9050	0.1511	7	0.3573	Median/MADe basis
	DerSimonian-Laird	2.6770	0.1946	7	0.4602	Between-lab SD: 0.522
	REML	2.6717	0.1777	7	0.4203	Between-lab SD: 0.472
	Weighted mean	2.5160	0.0412	7	0.0974	Weighted mean
	H15	2.7135	0.2584	7	0.6110	Huber (omitting u[i])
	Huber	2.6228	0.2044	7	0.4834	Huber (including u[i])

Note 1: Results and uncertainties are shown as copy number ratio, expressed as a percentage.

Note 2: Uncertainties cited here do not include an allowance for between-unit inhomogeneity of the test materials.

Note 3: "DF" is the degrees of freedom associated with the standard uncertainty *u*.

Note 4: The arithmetic mean is the simple average of reported results. The median is the median of reported results; MADe is the median absolute deviation scaled to provide a consistent estimate of standard deviation for a normal distribution. The DerSimonian-Laird (DSL) and REML approaches estimate an additional variance contribution, shown under "Remarks", that allows for dispersion that is not accounted for by the laboratories' reported measurement uncertainty. The DSL approximation is a simpler one-step calculation; REML is an iterative approach. The weighted mean is the Graybill-Deal weighted mean, using only the reported uncertainties for weighting. H15 is Huber's proposal 2, applied to the reported values but without using the reported uncertainties. "Huber" uses the same distance-dependent weighting function in a robust regression weighted by laboratory uncertainties.

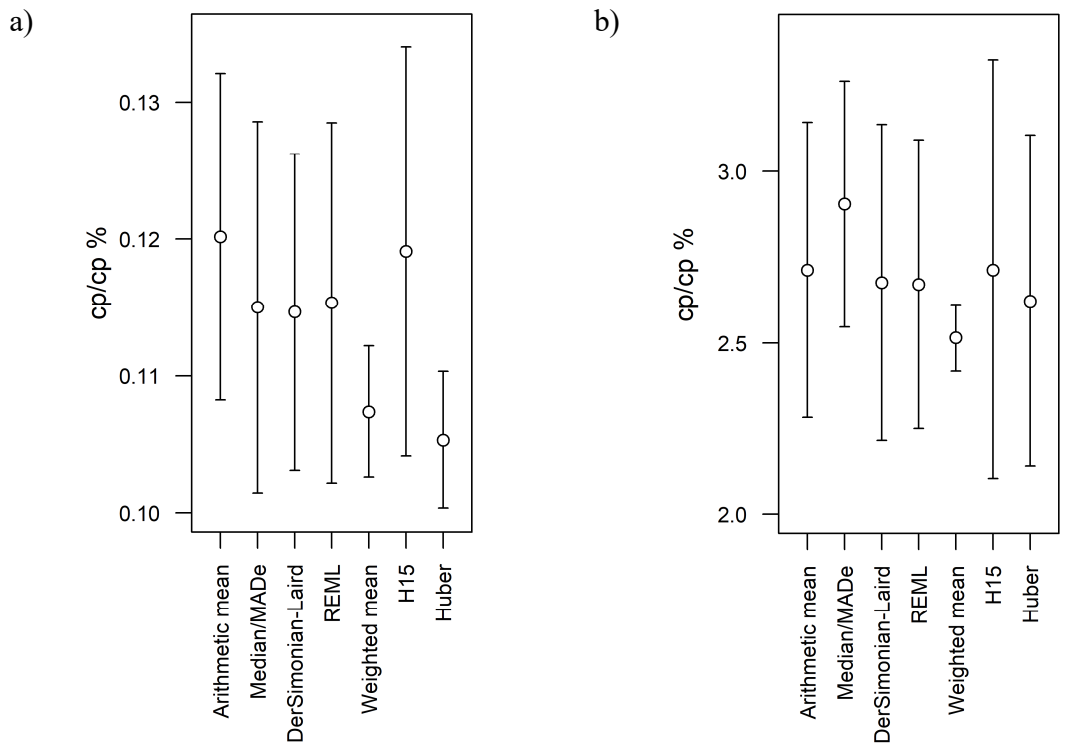

Figure 5. KCRV calculation approaches with expanded uncertainty

KCRV calculation approaches for a) sample T1 b) sample T2 (see Table 7).
Values are copy number ratio expressed as a percentage

The median was accordingly used for the calculation of the KCRV as decided by the WG. The median is **0.115 %** for sample T1 and **2.91 %** for Sample T2. Uncertainties associated with potential inhomogeneity were set at 5.2 % and 5.5 % (as relative standard uncertainties) for samples T1 and T2 respectively, giving contributions of 0.0568 % and 0.160 % in copy number ratio. The measurement uncertainty combined with the uncertainty introduced by the inhomogeneity of the samples resulted in combined expanded uncertainty of 0.017 % and 0.44 % for sample T1 and sample T2, respectively. The KCRVs, with expanded uncertainties, are shown in Figures 6 and 7 and in Table 8. Expanded KCRV uncertainties used a coverage factor k=2.

Table 8. KCRV for CCQM-K86.c – median based estimator

	KCRV[Note 1, Note 2] (cp/cp, %)	***u*** (cp/cp, %)	***U*** (cp/cp, %)
Sample T1	0.115	0.0086	0.017 (k=2)
Sample T2	2.91	0.22	0.44 (k=2)

Note 1: All values are given as copy number ratio, expressed as a percentage.

Note 2: The KCRV is given to three significant digits; the standard and expanded uncertainties to two.

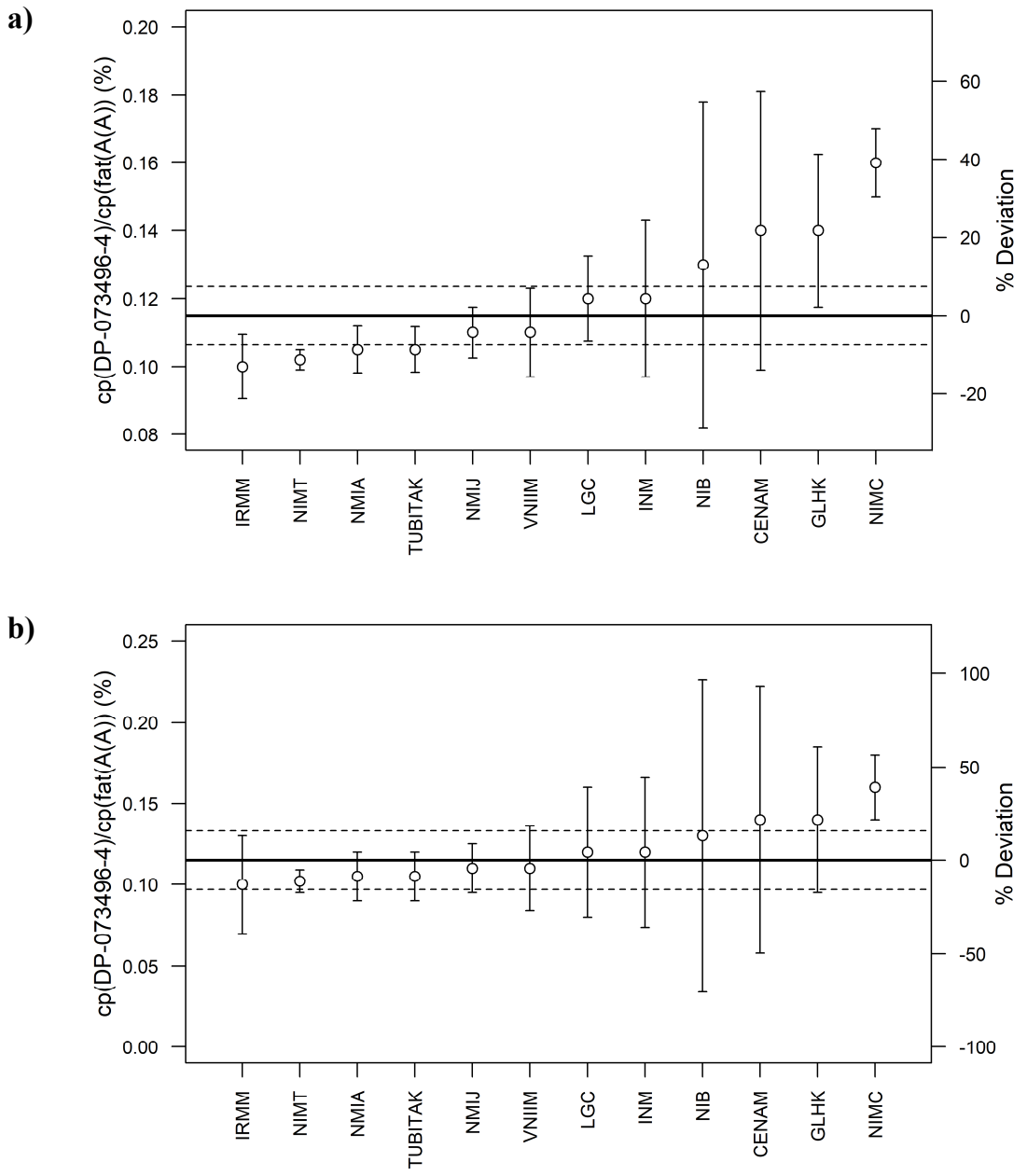

Figure 6. Reported results and uncertainties: sample T1. a) Standard uncertainties; b) expanded uncertainties.

All values are copy number ratios expressed as a percentage. Horizontal lines show the median as the Key Comparison Reference Value (solid line) with its uncertainty (dashed lines) using coverage factors a) k=1, b) k=2.23. Note the change in vertical scale from a) to b).

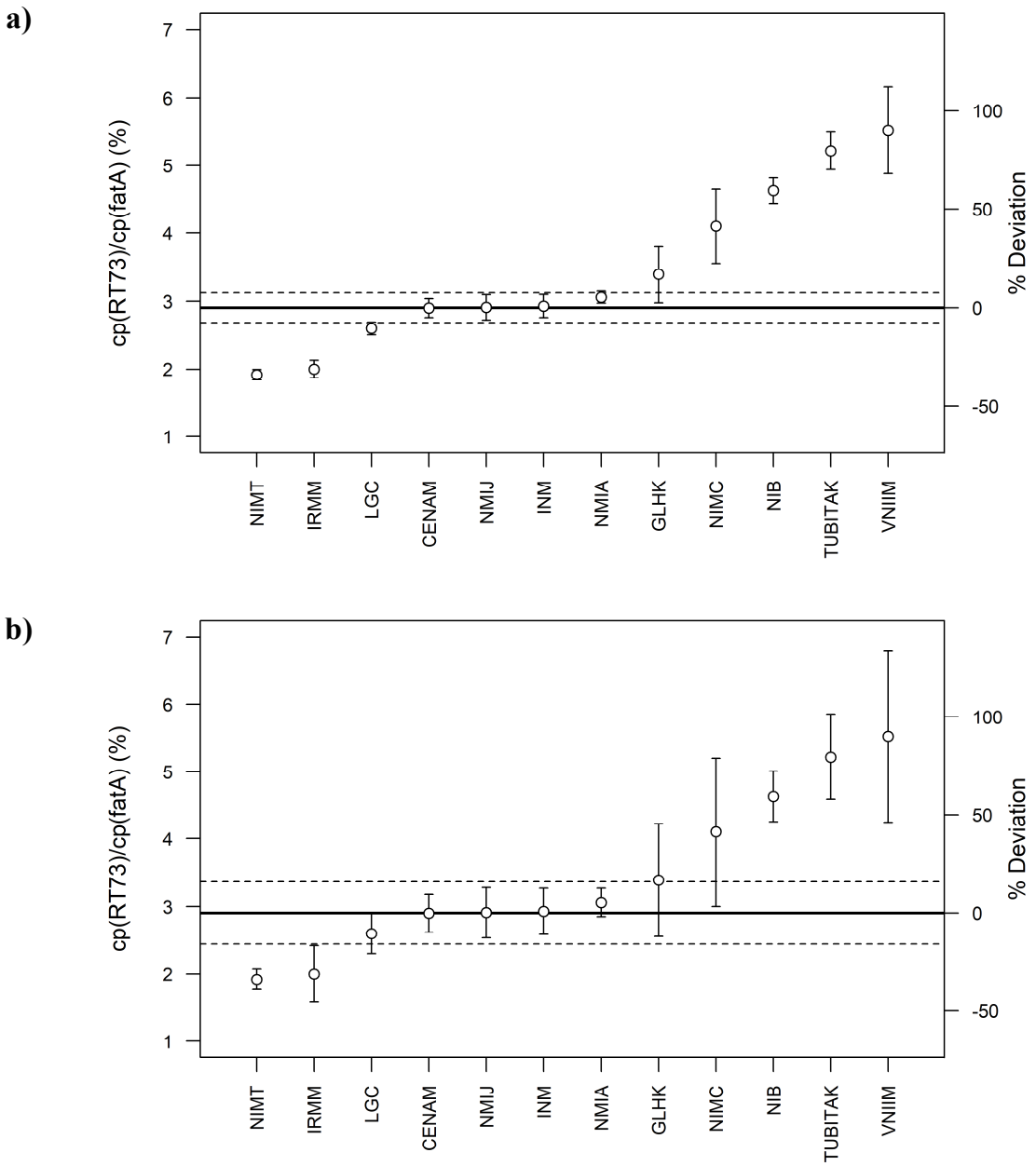

Figure 7. Reported results and uncertainties: sample T2. a) Standard uncertainties; b) expanded uncertainties.

All values are copy number ratios expressed as a percentage. Horizontal lines show the median as the Key Comparison Reference Value (solid line) with its uncertainty (dashed lines) using coverage factors a) k=1, b) k=2.45.

7.3 Degree of equivalence with respect to KCRV

The equivalence statements were calculated following CCQM guidance note CCQM13-22 on the calculation of degree of equivalence and associated uncertainty. [7] The resulting degrees of equivalence with expanded uncertainties are shown in Tables 9 and 10 and illustrated graphically in Figures 8 and 9.

Table 9. Degrees of equivalence (DoE) for sample T1[Note 1]

Participant	T1		
	D_i (cp/cp, %)	$u(Di)$ (cp/cp, %)	$U(D_i)$ ($k = 2$) (cp/cp, %)
IRMM	–0.015	0.013	0.026
NIMT	–0.013	0.009	0.018
NMIA	–0.010	0.011	0.022
TUBITAK	–0.010	0.011	0.022
NMIJ	–0.005	0.011	0.023
VNIIM	–0.005	0.016	0.031
LGC	0.005	0.015	0.030
INM	0.005	0.025	0.049
NIB	0.015	0.049	0.098
CENAM	0.025	0.042	0.084
GLHK	0.025	0.024	0.048
NIM China	0.045	0.013	0.026

Note 1. All values shown as copy number ratio, expressed as a percentage

Table 10. Degrees of equivalence (DoE) for sample T2[Note 1]

Participant	T2		
	D_i (cp/cp, %)	$u(Di)$ (cp/cp, %)	$U(D_i)$ ($k = 2$) (cp/cp, %)
NIMT	–0.99	0.23	0.47
IRMM	–0.91	0.26	0.51
LGC	–0.31	0.24	0.48
CENAM	–0.01	0.26	0.52
NMIJ	0.00	0.29	0.57
INM	0.02	0.28	0.56
NMIA	0.16	0.24	0.47
GLHK	0.49	0.47	0.94
NIM China[Note 2]	1.20	0.59	1.18
NIB[Note 2]	1.73	0.29	0.58
TUBITAK[Note 2]	2.32	0.36	0.71
VNIIM[Note 2]	2.62	0.68	1.35

Note 1. All values shown as copy number ratio, expressed as a percentage

Note 2. Reference gene: fatA(A)

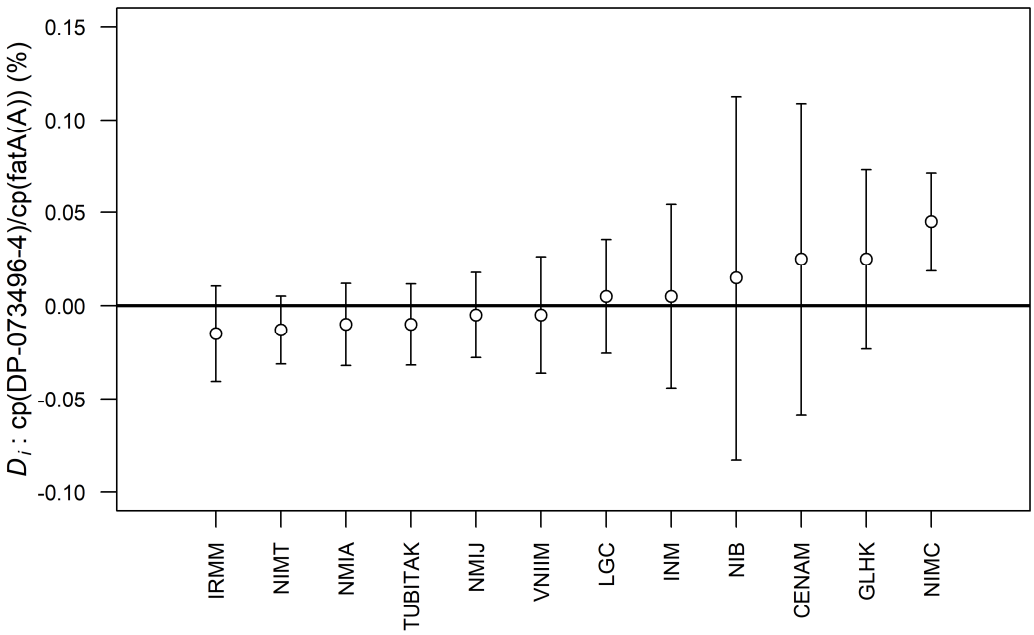

Figure 8. Degree of equivalence D_i with expanded uncertainties $U(D_i)$ (k=2) with respect to the KCRV for Sample T1

Note: Degrees of equivalence are shown in units of copy number ratio, expressed as a percentage, and not as a percentage of the KCRV

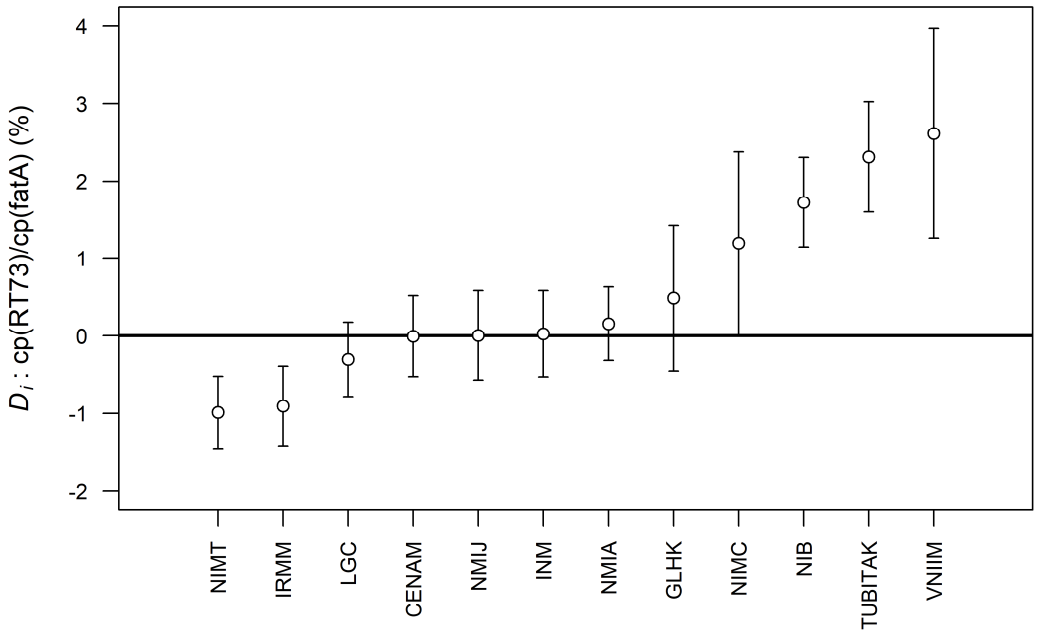

Figure 9. Degree of equivalence D_i with expanded uncertainties $U(D_i)$ (k=2) with respect to the KCRV for Sample T2 (all participants).

Note: Degrees of equivalence are shown in units of copy number ratio, expressed as a percentage, and not as a percentage of the KCRV

8 Discussion

Considering the complexity of the analytical challenge a reasonable level of agreement between the laboratories was observed. The only significant deviation observed was related to choice of reference gene; four laboratories for the analysis of sample T2 chose fatA(A) as their reference gene instead of the suggested use of fatA.

Brassica napus L. species originates from the crossing between *B. oleracea* (e.g. broccoli, cabbage) and *B. rapa* (e.g. Pak Choi, Chinese cabbage) [8]. *B. napus* is called an amphidiploid species; it has 10 chromosomes from *B. rapa* (A genome) and 9 chromosomes from *B. Oleracea* (C genome). Due to its origin, some gene loci (in particular, fatA) exist as duplicates in the genome of *B. napus*. This is the case for the fatA gene coding for oleoyl hydrolase which is present as single copy on both the A and C genomes. The gene fatA(A) is only present as a single copy in the A genome and is not present on the C genome (see, for example, [9]).

Because of this, the four laboratories using fatA(A) as a reference gene for sample T2 were not considered for KCRV calculation. Degrees of equivalence were assigned for these laboratories after calculating the KCRV based on the remaining participant results. We note, however, that the data presented by the four laboratories were in good agreement among themselves. During the working group evaluation stage it was considered whether a simple correction factor of 2, reflecting the number of copies of the reference sequences in the genome, could be used to correct the data for these labs but this proposal was rejected because several untested assumptions would have to be made to support this action.

9 Conclusions

CCQM-K86.c supports measurement claims for the quantification of the ratio of the number of copies of specified intact sequence fragments of a length up to 150 nucleotides following extraction from an unprocessed, high fat/oil ground seed matrix, with a copy number ratio from 0.001 to 1.

The complexity of analyzing animal tissue, cell culture or other similar samples represent a lower level of complexity than the plant based samples studied here, so this study can also

be used as evidence of a general capability for DNA quantitation in those areas. Obviously the farther the measurement claim is from this exact comparison the more additional evidence is needed.

10 References

1. P. Corbisier, S. Vincent, H. Schimmel, A-M Kortekaas, S. Trapmann, M. Burns, C. Bushell, M. Akgoz, S. Akyürek, L. Dong, B. Fu, L. Zhang, J. Wang, M. Pérez Urquiza, J L. Bautista, A. Garibay, B. Fuller, A. Baoutina, L. Partis, K. Emslie, M. Holden, W. Y. Chum, H-H Kim, N. Phunbua, M. Milavec, J. Zel, M. Vonsky, L. A. Konopelko, T. L. T. Lau, B. Yang, M. H. K. Hui, A. C. H. Yu, D. Viroonudomphol, C. Prawettongsopon, K. Wiangnon, R. Takabatake, K. Kitta, M. Kawaharasaki, H. Parkes (2012) *CCQM-K86/P113.1: Relative quantification of genomic DNA fragments extracted from a biological tissue*. Metrologia, **49,** 1A 08002 http://stacks.iop.org/0026-1394/49/i=1A/a=08002

2. L. Dong, Z. Sui, J. Wang, V. H. M Tang, W. W. Y. Chum, F-W Lee, D. W. M. Sin, M. Pérez–Urquiza, M. Burns, S. L. R. Ellison, H. Parkes, M. Milavec, C. Prawettongsopon, K. R. Griffiths, J. L. H. McLaughlin, S. Shibayama, S. Akyurek and M. Akgoz. (2018) *Final report for CCQM-K86.b relative quantification of Bt63 in GM rice matrix sample* Metrologia 55 (1A) 08017. http://stacks.iop.org/0026-1394/55/i=1A/a=08017

3. B. Dimitrievska, A.M. Kortekaas, M. Contreras, J. Charoud-Got, P. Conneely, H. Emteborg, P. Corbisier, S. Trapmann (2013) Certification report. The certification of different mass fractions of the GM event 73496 in rapeseed powder. doi:10.2787/82091

4. NRC Canada, in-house data, accessed on September 2017

5. European Commission Joint Research Centre (2014) Event-specific Method for the Quantification of Oilseed Rape DP-073496-4 Using Real-time PCR; EURL-VL-02/12VP corrected version 1. http://gmo-crl.jrc.ec.europa.eu/summaries/EURL-VL-02-12VP-EFSA-Corr1.pdf

6. S. Holm (1979). *A simple sequentially rejective multiple test procedure*. Scand. J. Stats **6**, 65-70.

7. CCQM13-22. CCQM Guidance note: Estimation of a consensus KCRV and associated Degrees of Equivalence, Version: 10, (2013). https://www.bipm.org/cc/CCQM/Allowed/19/CCQM13-22_Consensus_KCRV_v10.pdf,

8. R. K. Downey and S. R. Rimmer (1993). Agronomic Improvements in Oilseed Brassicas. Advances in Agronomy 50:1-66.

9. N. Henderson, M. Harmon, C. X. Zhong (2016) PCR-Based Detection and Quantification of a Transgenic Glyphosate-Tolerant Canola Using a Novel Reference Gene System. Food Anal. Methods 9: 353. https://doi.org/10.1007/s12161-015-0156-0

Appendix A: Study protocol[1]

CCQM-KC86.c: Key comparison "Relative quantification of genomic DNA fragments extracted from a biological tissue"
Study Protocol

Introduction

The quantification of the relative abundance of two DNA fragments, one being present between in ratio of 1/1000 to 1/10 in a difficult matrix such as plant material has been demonstrated in previous Key Comparisons (CCQM-K86 and CCQM-K86.b).

The matrix in CCQM-K86 was a maize seed powders, whereas in CCQM-K86.b it was a rice seed powder. Both matrixes are rich (between 75 and 80 g/100g) in polymeric carbohydrate (amylose and amylopectine) and poor in fat (<5 g/100g) requiring adapted DNA extraction method to remove substances that can hinder the polymerase activity.

In this study, the NAWG decided to challenge laboratories with a plant material rich (40 g/100g) in fat, such as rapeseed. As the rapeseed is genetically engineered, validated DNA extraction and qPCR quantification methods are available.

Description of the Measurands

Both samples to be analysed are rapeseed powders each containing a low but different number of copies of the same defined DNA sequences.

Preparation of Unknown Samples

Sample T1, one rapeseed powder containing a defined mass fraction of genetically modified

[1] The study protocol is reproduced as distributed to participants *except that* abbreviated references to other studies (e.g. "K86b") have been amended to full references (e.g. "CCQM-K86.b") for consistency with the body of this report, and page numbers continue from the body of the report.

(GM) DP-Ø73496-4 rapeseed (*Brassica napus* L.). The samples were produced under the responsibility of the JRC and were prepared by mixing of dried non-GM rapeseed powder and DP-Ø73496-4 GM dried rapeseed powder.

Sample T2, one rapeseed powder containing a defined mass fraction of genetically modified (GM) GT73/RT73 rapeseed (*Brassica napus* L.). The samples were produced under the responsibility of NRC and were prepared by mixing of dried non-GM rapeseed powder and GT73/RT73 dried rapeseed powder.

PCR assays

GM rapeseed can be assayed by adapting a qPCR method validated by the European Union reference Laboratory for GM food and feed (EURL-GMFF) [1,2] and/or Monsanto Regulatory Sciences method[3] to digital PCR or similar approaches.

No calibrant for the quantification of the unknown samples by qPCR will be provided.

Sample T1

For the specific detection of the DP-Ø73496-4 event, a 84 bp fragment that spans the 5' junction between the DP-Ø73496-4 insert and the flanking rapeseed genomic DNA is amplified using two specific primers and a probe. The forward primer is situated within the rapeseed genomic DNA, the reverse primer is situated within the inserted DNA and the probe spans the transition between the DP-Ø73496-4 insert and rapeseed genomic DNA.

For the specific detection of the rapeseed reference, a 126 bp fragment of the *fatA(A)* gene is amplified using two specific primers and a probe. This rapeseed specific PCR system was designed to detect the oleoyl hydrolase *fatA(A)* gene from *Brassica rapa*, *Brassica napus* and *Brassica juncea*. It is used as reference system for relative quantification of event DP-

[1] http://gmo-crl.jrc.ec.europa.eu/summaries/EURL-VL-02-12VP-EFSA-Corr1.pdf

[2] http://gmo-crl.jrc.ec.europa.eu/summaries/RT73_validated_Method.pdf

[3] http://gmo-crl.jrc.ec.europa.eu/detectionmethods/MON-Art47-pcrGT73rapeseed.pdf

Ø73496-4.

The ratio between the copy number of those two DNA sequences in the sample must be determined:

Target sequence 1: (84 bp)
GTTCTTCTCTTCATAGCTCATTACAGTTTTCATTAGTTAGATCAGGATATTCTTGT
TTAAGATGTTGAACTCTATGGAGGTTTG

Target sequence 2 (126 bp):
ACAGATGAAGTTCGGGACGAGTACTTGGTTTTCTGTCCTCGAGAACCCAGGTGA
AGAAGAATCATCATGCTTCCCTTATAATTGCTAGTTAAACAGTTAATATTTAAGC
ATGTGGATCTCAACCTG

Note: the amplified *fatA(A)* fragment is of 126 bp in a majority of *Brassica napus* varieties, in all *Brassica juncea* varieties and in some of the *Brassica rapa* varieties tested; it is of 129 bp in a minority of *Brassica napus* varieties and in some Brassica *rapa* varieties tested.

Participants should determine the absolute number of both DNA targets by digital PCR and to provide the ratio of those two numbers.

Sample T2
Forward Primer
Name: RT73 primer 1
Sequence: CCATATTGACCATCATACTCATTGCT
Size: 26 bases

Reverse Primer
Name: RT73 primer 2
Sequence: GCTTATACGAAGGCAAGAAAAGGA

Size: 24 bases

Probe

Name: RT73 Probe

Sequence: TTCCCGGACATGAAGATCATCCTCCTT

Size: 27 bases

Amplicon

Size: 108bp

Forward Primer

Name: FatA primer1

Sequence: GGTCTCTCAGCAAGTGGGTGAT

Size: 22 bases

Reverse Primer

Name: FatA primer2

Sequence: TCGTCCCGAACTTCATCTGTAA

Size: 22 bases

Probe

Name: FatA probe

Sequence: ATGAACCAAGACACAAGGCGGCTTCA

Size: 26 bases

Amplicon

Size: 76 bp

Information on the RT73 assay on the proposed fatA amplicon could be found in ref 1 and 2. Participants should determine the absolute number of both DNA targets by digital PCR and to provide the ratio of those two numbers.

Participants have the possibility to use any other type of calibrant which they think enables them to report a copy number ratio, expressed in percent, between the DP-Ø73496-4 / RT73 and *fatA(A)* fragments. In this study two different fatA fragments are being suggested for the two samples. The reason being that the samples were assessed only with these amplicons.

Homogeneity testing

Homogeneity analysis of the DNA sequences in sample T1 and 2 has been performed by JRC and NRC and the uncertainty related to the homogeneity will be provided. The sample intake used for determining the homogeneity was 200 mg.

Stability testing

For sample T1 JRC performed a short-term stability study was carried out using an isochronous design. In this approach, samples were stored for a certain time at different temperature conditions. Afterwards, the samples were moved to conditions where further degradation can be assumed to be negligible (reference conditions). At the end of the isochronous storage, the samples are analysed simultaneously under intermediate precision conditions. The study concluded that the samples can be dispatched without further precautions under ambient conditions.

The CRM ERM-BF434e (containing 100 g of DP-Ø73496-4 rapeseed per kg of rapeseed powder) has been produced at the same time and under the same conditions as the CRM ERM-BF434c (which is sample T1 and contains 1 g of DP-Ø73496-4 rapeseed per kg of rapeseed powder). The stability of CRM ERM-BF434e has been monitored since the production in 2013 and was found stable at 4°C over a period of 6 years (Fig 1A) which suggests that the stability of T1 produced and kept under the same conditions is assured.

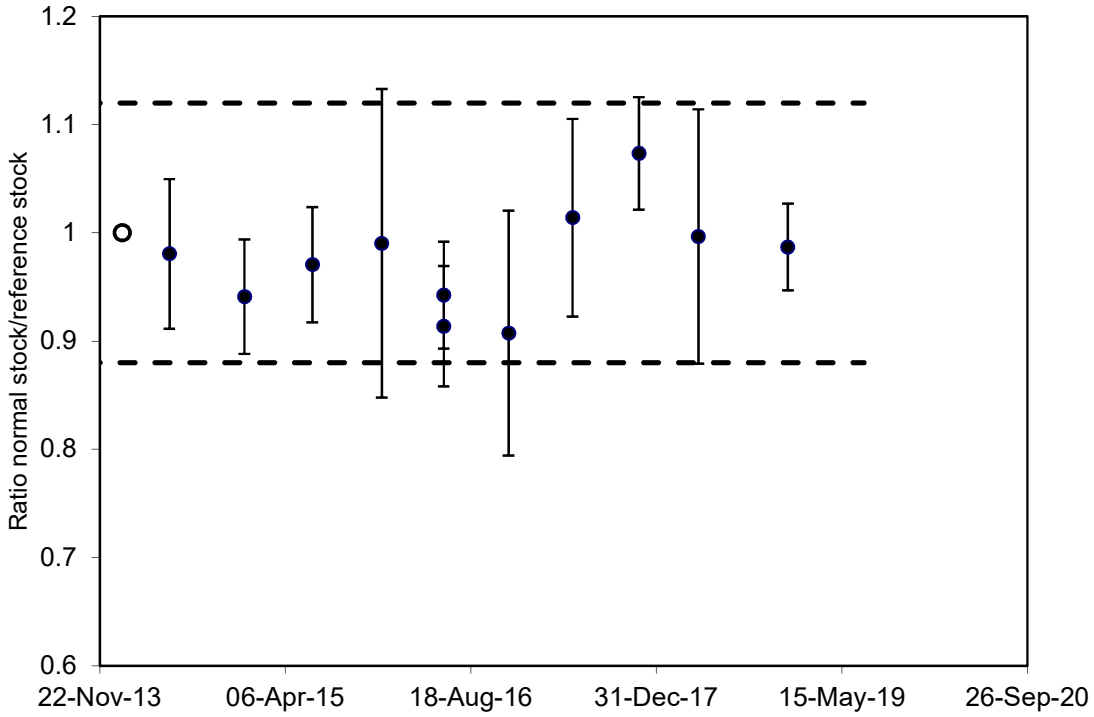

Figure A1: Long term stability of ERM-BF434e kept at 4°C.

Note 1 : **Each points represent the average ratio of the** DP-Ø73496-4/FatA(A) content of samples kept at 4°C (normal stock) and at -70°C (reference stock) **over a period of 6 years.**

Note 2: Errors bars rep**resent the expanded uncertainty (*k*=2) associated to the qPCR results and** the upper and lower certified ranges are given by the dashed **lines.** The **certified value divided by the certified value** at time point 0 (**22 Jan 2014**) is given by an open circle**.**

Sample T2 has been found stable for over 10 years (data not shown).

Instructions for use

Participants will receive 2 glass bottles each containing at least 1 g of samples 1 and 1g of sample T2. Samples 1 and 2 should be stored at 4 ºC.

Methodology

Sample T1 will be dispatched from JRC sample T2 will dispatched from NRC Canada.
Participants are requested to use their preferred methodology for the extraction of genomic DNA from samples 1 and 2. Special care should be taken to prepare sufficiently purified genomic DNA. The unknowns can be diluted to verify the absence of PCR inhibitors in the extracted DNA. For example, the extraction method referred to by the European Union reference Laboratory for GM Food and Feed can be applied[3].
Digital PCR is the preferred method to quantify DNA sequences when calibrant solution are not existing but other technologies can also be used. The digital PCR platform and chemistry can be chosen by the participants.

Reporting

A reporting template and detailed questionnaire will be provided to the participants. The participants shall submit an electronic version of the reporting template and questionnaire by e-mail to Zoltan.Mester@nrc-cnrc.gc.ca. In addition, a signed and dated copy of the report shall be sent by surface mail to the address mentioned below or as a PDF-file by e-mail to Zoltan.Mester@nrc-cnrc.gc.ca. The participants shall burn a copy of the report and of the raw data on a CD-ROM or memory stick.

The results indicating the relative percentage of both sequences present should be reported for each DNA extraction of the unknown replicates as well as the stated uncertainty. An overall combined result for each sample should also be included.

All results returned should include,

- The absolute copy number of both sequence targets for samples 1 and 2 per µL DNA in the PCR MasterMix

[3] http://gmo-crl.jrc.ec.europa.eu/summaries/EURL-VL-02-12XP-EFSA.pdf

- The ratio of both sequence targets expressed in percent for samples 1 and 2 as well as the uncertainty
- An outline of the methodology, a measurement equation and a breakdown of the uncertainty estimation submitted.

Please report results by e-mail to:

Dr Zoltan Mester
National Research Council Canada
1200 Montreal Rd,
Ottawa, ON, K1W1E4
CANADA
Phone: +1 613 993 5008
Fax: +1 613 993 2451
E-mail: zoltan.mester@nrc-cnrc.gc.ca

Proposed Comparison Timetable

Deadline for signup to the study[4]: November 11, 2016
Distribution of sample materials: November 28, 2016
Deadline for submission of results: March 31 2017
preliminary report: NAWG meeting, April 2017
Draft A report one month after the NAWG meeting
Final Report Fall NAWG meeting 2017

[4] The study co-ordinator is requesting that, in view of the cost of the samples, participants should commit to submission of results within the study deadline.