CCQM-K86/P113.1: Relative quantification of genomic DNA fragments extracted from a biological tissue

Draft B

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Summary

Key comparison CCQM-K86 was performed to demonstrate and document the capacity of interested National Metrology Institutes (NMIs) and Designated Institutes (DIs) in the determination of the relative quantity of two specific genomic DNA fragments present in a biological tissue.
The study provides the support for the following measurement claim:

"Quantification of the ratio of the number of copies of specified intact sequence fragments of a length in the range of 70 to 100 nucleotides in a single genomic DNA extract from ground maize seed materials ".

The study was carried out under the auspices of the Bioanalysis Working Group (BAWG) of the Comité Consultatif pour la Quantité de Matière (CCQM) and was piloted by the Institute for Reference Materials and Methods (IRMM) in Geel (Belgium).

The following laboratories (in alphabetical order) participated in this key comparison.

AIST (Japan); CENAM (Mexico); DMSc (Thailand); GLHK (Hong Kong); IRMM (European Union); KRISS (R. of S. Korea); LGC (United Kingdom); MIRS/NIB (Slovenia); NIM (P.R. of China); NIST (USA); NMIA (Australia); TÜBİTAK UME (Turkey); VNIIM (Russian Federation).

The following laboratories (in alphabetical order) participated to a pilot study that was organised in parallel.

LGC (United Kingdom); PKU (P.R. of China); NFRI (Japan); NIMT (Thailand).

Good agreement was observed between the reported results of eleven participants.

Contents

EDITORIAL NOTE

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1 Introduction

The provision of traceable standards to the biological community is an area of active research in many NMIs or DIs. The quantification of the relative amount of DNA sequences extracted from a biological tissue remains a complex analytical procedure and relies on the availability of such standards. Real-time quantitative polymerase chain reaction (qPCR) is currently the most applied measurement method to identify and quantify DNA sequences. Several NMIs and DIs were able to demonstrate their ability to use this technology to quantify a defined plasmid DNA using the same plasmid DNA as a calibrant (CCQM-P44 (1&2) and CCQM-K61). The same measurement method was used to quantify genomic DNA extracted from a plant tissue and calibrated by a genomic DNA solution extracted from the same plant material (CCQM-P60). In a later study, the importance of a reliable DNA extraction method was underlined. The analytical procedure was more complex in CCQM-P60 compared to CCQM-K61, as it included a DNA extraction step. The main objective of CCQM-K61 was to establish international comparability in the quantitation of a linearised plasmid DNA, based on a matched standard in a matrix of non-target DNA. However, both studies were performed using matching calibrants for which a reference value had been assigned.
The pilot study CCQM-P113 has been organised to demonstrate the ability to quantify DNA sequences present in a biological tissue using an independent calibration system. The quantification was largely performed by qPCR. 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 organise two studies in parallel: a key comparison, CCQM-K86 and a pilot study, CCQM-P113.1 based on materials provided by IRMM.

2 Rationale of this comparison

The CCQM-K86 is the second key comparison carried out under the auspices of the BAWG. The main aim was to underpin Calibration and Measurement Capabilities (CMCs) for the determination of the relative quantity of two genomic DNA fragments present in a biological tissue. The ratio between two genomic DNA fragments has been determined mainly by qPCR but 3 laboratories have submitted their results based on digital PCR (dPCR) measurements. With the first method, the fluorescent signal which is generated during the amplification was calibrated with an external calibrant whereas no calibrant is needed when dPCR is performed. The ratio between absolute numbers of copies of both genomic DNA fragment was determined in the latter case.

3 Measurement Claim

The measurement claim for CCQM-K86 is:

"Quantification of the ratio of the number of copies of specified intact sequence fragments of a length in the range of 70 to 100 nucleotides in a single genomic DNA extract from ground maize seed materials".

The study supports the participants' competence to extract DNA from ground maize seed materials and to perform measurements on the extracted DNA using qPCR where an independent reference material is used as calibrant. The study supports also the competence of participants to determine the ratio of two DNA targets using dPCR measurements.

The matrix is defined as maize seed materials. The samples were composed of ground seed material requiring optimized DNA extraction method. The samples analysed were however already ground which means that neither the grinding procedure nor a particular sampling plan are supported by this key comparison.

The KC may additionally be used to support claims related to copy number ratio determination in a wider range of biological tissues subject to additional evidence of effective extraction and clean-up procedures. The KC shall not be used to support claims requiring quantitative extraction of genomic DNA.

The measurand is the ratio of number of copies of specified intact sequence segments of a length in the range of 70 to 100 nucleotides in a single genomic DNA extract.

The dissemination range of measurement capability goes from 0.3 to 2.5 (copy number ratio expressed in %). The materials tested were also assigned a mass fraction based on the gravimetric dry-mixing of non-GM maize powder with MON810 maize seed powder. The assigned values were 8.1 ± 0.7 g/kg and 38.3 ± 1.7 g/kg (k = 2) for sample 1 and 2, respectively.

The qPCR results are traceable to a calibration material certified for a copy number ratio of 1 with negligible uncertainty (such as a plasmid containing one copy of each sequence segment).

For dPCR, the traceability is to unity (provided the sequence segment appears only once per genome).

For both qPCR and dPCR results, the identity of the measurand is operationally defined as only DNA molecules that are amplified or "PCR active" under the specified experimental conditions can be measured or counted.

The determination of the ratio by qPCR was realised by using either plasmid calibrant (ERM-AD413) or a matrix material (ERM-BF413d) both certified for their copy number ratio. The ERM-AD413 is a certified reference material (CRM), the certified value is expressed as a number of specific DNA fragments per plasmid. The number is determined on the basis of the sequence of the plasmid and is traceable to the International System of Units (SI). The ERM-BF413d is a maize powder material, the certified value is based on the maize MON810 DNA copy numbers and taxon-specific DNA copy numbers. The DNA copy number ratio has been determined using the MON810 event-specific method calibrated with the maize plasmid DNA (pDNA) CRM ERM-AD413. Given the procedure and calibrant, the certified value is traceable to the SI.

The CCQM-K86 did not cover the design and optimization of primers and probe as these information was provided to the participants. The samples analysed in the CCQM-K86 were ground samples from which high molecular weight genomic DNA could be extracted. The current CCQM-K86 can therefore not be used to claim genetically modified (GM) detection in highly processed food or feed products from which low molecular weight genomic DNA can be retrieved. The quantification of GM events other than MON810 but based on qPCR can nevertheless be supported by this key comparison provided that an appropriate calibrant is available for the qPCR procedure applied.
Several different extraction methods have been applied to extract the DNA from the maize tissues leading to very similar final results. However, the contingent change in the composition of a commercial DNA extraction kit used in CCQM-K86 or the removal of that kit from the market could impair the claim based on such extraction kit.

4 Participation in CCQM-K86

The 13 NMIs or DIs that participated in CCQM-K86 are listed in Table 1. In addition, there were three participants in a parallel pilot study CCQM P113.1 (Table 2). One participant also decided to submit its results obtained by dPCR in the frame of the CCQM P113.1 study. The affiliation names of the participants of the pilot study are not mentioned in the results tables and figures.
References to a protocol (Appendix D) were sent to all participants prior to sample distribution and 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 on the two unknown samples as received. IRMM coordinated the study.

Table 1: CCQM-K86 participants

Institute / Organisation	*Country*	*Contact*
IRMM – Institute for Reference Materials and Measurements	European Union	P. Corbisier
LGC	United Kingdom	M. Burns
TÜBİTAK UME - Ulusal Metroloji Enstitüsü	Turkey	M. Akgoz
NIM - National Institute of Metrology	P. R. China	W. Jing
CENAM - National Metrology Center	Mexico	M. Pérez Urquiza
NMIA - National Measurement Institute Australia	Australia	A. Baoutina
NIST - National Institute of Standards and Technology	USA	M. Holden
GLHK - Government Laboratory Hong Kong	Hong Kong	W. Y. Chum
KRISS - Korea Research Institute of Standards and Science	R. of S. Korea	H.-H. Kim
DMSc - Department of Medical Sciences	Thailand	N. Phunbua
MIRS/NIB - National Institute of Biology	Slovenia	M. Milavec
VNIIM - D.I. Mendeleev Institute for Metrology	Russian Federation	M. Vonsky
AIST – National Institute of Advanced Industrial Science and Technology	Japan	M. Kawaharasaki

Table 2: CCQM-P113.1 participants

Institute / Organisation	*Country*	*Contact*
LGC	United Kingdom	M. Burns
PKU - Peking University	P.R. China	A. C. H. Yu
NFRI – National Food Research Institute	Japan	R. Takabatake
NIMT - National Institute of Metrology Thailand	Thailand	D. Viroonudomphol

5 Test material preparation

The two unknowns consist of non-GM and GM MON810 maize seed powders. The samples produced under the responsibility of the IRMM were prepared by mixing dried non-GM maize seed powder and dried GM MON810 maize seed powder. Details of the test material preparation and value assignment are given in Appendix A.
The two unknown samples were checked for homogeneity and stability by the coordinating laboratory during the time the participants conducted the analysis.

6 Methods and instrumentation used

Participants were free to use a method of their choice for the quantitative determination of MON810 maize. A method was nevertheless provided describing the detection and amplification of a specific DNA target for MON810 and a DNA target for maize.

For the specific detection of GM MON810 maize a 92 bp fragment of the single copy DNA integration-border region of the genomic sequence and the inserted sequence element originating from CaMV (35S promoter) as a result of in vitro recombination present in the GM insect-protected MON810 maize is amplified.
For relative quantification of MON810 maize, a 79 bp fragment of the taxon-specific maize high mobility group protein gene (*hmg*) is amplified.

For quantification of the targets a calibrant such as the ERM-AD413 was suggested to be used. The ERM-AD413 was processed and certified according to the ISO Guide 34 and is available for sale [1]. The CRM is certified for the number of DNA fragments per plasmid of a MON810 transgenic sequence and of the *hmg*. ERM-AD413 is intended for the calibration of MON810 maize PCR method as described and validated by the European Union Reference Laboratory for GM Food and Feed (EURL-GMFF) [2].

The ratio between the copy number of the two DNA sequences in the samples 1 and 2 had to be determined:

Target sequence 1 (92 bp)
gCCACCTTCCTTTTCCACTATCTTCACAATAAAgTgACAgATAgCTgggCAATggCAAAggATgTTAAACgTTAgAgTCCTTCgTCCTTCgA

Target sequence 2 (79 bp)
GCTACATAgggAgCCTTgTCCTACAATCCACACAAACgCACgCgTAAAACAATTAATCAgCACgAgATTTCTAgTCCAA

Participants had the possibility to use any other type of calibrant to report a copy number ratio, expressed in percent, between the MON810 and *hmg* fragments measured.

Participants had also the possibility to measure the absolute number of both DNA targets by dPCR and to provide the ratio of those two numbers.

The majority of participants carried out their measurements by qPCR; three participants reported their results using dPCR. Table 3 gives an overview of the extraction methods, instrumentation, the chemistries, amplicons sizes as well as the nature of the calibrant or quality control materials used by the participants.

Three participants have used the same chemical DNA extraction method (CTAB), the other participants have all extracted DNA using different DNA extraction kits. The most used qPCR apparatus in the study were the Sequence Detection Systems developed by Applied Biosystems (models 7500 and 7900HT). The models differ mainly in the nature of their excitation source (a 488 nm argon-ion laser and a tungsten-halogen lamp as excitation source for the 7900 and 7500, respectively). One participant performed the reactions using 384 wells rather than the classical 96 well format. Two different Light Cycler models have been used either in 96 well plates (Roche LC 480) or using the 32 glass capillaries (Roche LC 2.0) format. VNIIM used a Russian qPCR apparatus referenced as ANK64.
The absolute quantification of DNA targets was performed by three participants using the Biomark system from Fluidigm.
Most participants used the TaqMan Universal PCR Master Mix as recommended in the protocol. Specific Master Mixes were used for both Roche and the ANK64 apparatus used in this study. One laboratory used TaqMan Gene Expression Master Mix rather than the TaqMan Universal PCR Master Mix.
The probes were dual labelled mainly using FAM and TAMRA. In some cases TAMRA was replaced by non-fluorescent Black Hole Quenchers (BHQ-1) or Minor Groove Binder (MGB) quenchers; their advantage is to generate lower background signal. FAM was replaced by Cal Fluor Orange (CLO560) in one case as an alternative to VIC fluorescent probe.
All participants have amplified the 79 bp amplicon for the *hmg* taxon-specific gene. Two participants have used another reference gene (*zSSIIb*) present in the Nippon gene calibrator (pMuI5) [3]. Most participants have amplified the 92 bp amplicon for MON810. Two participants also amplified a 113 bp fragment of MON810, whilst two participants only amplified either a 70 bp or 115 bp fragment. ERM-AD314 certified reference material was the most commonly used calibrant for the qPCR, however two participants also used in parallel the Nippon gene calibrant (pMuI5). One participant used a powder material certified by IRMM for its MON810 copy number percentage (ERM-BF413d) to generate calibration curves. CENAM used an in-house MON810 reference material which is certified for its mass content. A small number of laboratories used other certified reference materials produced by IRMM as quality control or to evaluate bias.

Institutes	Extraction method	Instrument	Reagents	Probe MON810	Probe reference	MON810 Amplicon size (bp)	Reference amplicon size (bp)	Calibrant	QC
AIST	Wizard Magnetic Purification kit	ABI 7900	TaqMan® Universal PCR Master Mix	FAM/MGB	FAM/TAMRA	70	79	ERM-AD413	
CENAM	Genetic ID kit	ABI 7900 (Fast mode)	TaqMan® Universal PCR Master Mix	FAM/TAMRA	FAM/TAMRA	92	79	CENAM RM, DMR 436 series Ia-Va	ERM-BF413f; ERM-BF413c
DMSc	Wizard Genomic DNA extraction kit	Roche LC 2.0	LC® FastStart DNA Master HybProbe	FAM/TAMRA	FAM/TAMRA	92	79	ERM-AD413	
GLHK	CTAB + MaXtract™ kit	ABI 7500	TaqMan® Universal PCR Master Mix	FAM/TAMRA	FAM/TAMRA	92	79	ERM-AD413	
IRMM	GENE*Spin*	ABI 7900HT	TaqMan® Universal PCR Master Mix	FAM/TAMRA	FAM/TAMRA	92	79	ERM-AD413	
IRMM (d)	GENE*Spin*	dPCR Biomark Fluidigm	TaqMan® Universal PCR Master Mix	FAM/TAMRA	FAM/TAMRA	92	79	not needed	
KRISS	CTAB	ABI 7900HT	TaqMan® Universal PCR Master Mix	FAM/TAMRA	FAM/TAMRA	92 113	79 151	ERM-AD413 pMul5	ERM-BF413f
Lab 1 (d)*	CTAB	dPCR Biomark Fluidigm	TaqMan® Universal PCR Master Mix	FAM/TAMRA	FAM/TAMRA	92	79	not needed	ERM-BF413b
Lab 2.1*	GM quicker extraction kit	ABI 7900HT	TaqMan® Universal PCR Master Mix	FAM/TAMRA	FAM/TAMRA	113	114	pMul5	
Lab 2.2*	GM quicker extraction kit	ABI 7900HT	TaqMan® Universal PCR Master Mix	FAM/TAMRA	FAM/TAMRA	92	79	ERM-AD413	
Lab 3*	Hai Kang Life Food Ex DNA extraction kit	ABI 7500	TaqMan® Gene Expression Master Mix	FAM/TAMRA	FAM/TAMRA	92	79	ERM-AD413	
Lab 4*	Qiagen Dneasy Plant Mini kit	ABI 7500	TaqMan® Universal PCR Master Mix	FAM/TAMRA	FAM/TAMRA	92	79	ERM-AD413	
LGC	CTAB	ABI 7900	TaqMan® Universal PCR Master Mix	FAM/TAMRA	FAM/TAMRA	92	79	ERM-AD413	
MIRS/NIB	NucleoSpin Food kit	ABI 7900 (Fast mode) 384	TaqMan® Universal PCR Master Mix	FAM/TAMRA	FAM/TAMRA	115	79	ERM-BF413d	
NIM	TIANGEN Plant DNA extraction kit	ABI 7900	TaqMan® Universal PCR Master Mix	FAM/TAMRA	FAM/TAMRA	92	79	ERM-AD413	
NIST	CTAB + S-300 HR MicroSpin Columns	ABI 7500	TaqMan® Universal PCR Master Mix	FAM/BHQ-1	CLO560/BHQ -1	92	79	ERM-AD413	ERM-BF413d
NMIA (d)	Wizard Genomic DNA extraction kit	dPCR Biomark Fluidigm	Taqman® Universal Master Mix (+ AmpErase UNG)	FAM/BHQ-1	FAM/BHQ-1	92	79	not needed	ERM-BF413
TÜBİTAK UME	Qiagen Dneasy Plant Mini kit	Roche LC 480	LC® 480 Probes Master	FAM/TAMRA	FAM/TAMRA	92	79	ERM-AD413	
VNIIM	Sorb-GMO- B kit	ANK64	ZAO "Syntol"	FAM/BHQ-1	FAM/BHQ-1	92	79	ERM-AD413	

Table 3: Analytical methods and instrumental techniques used by the CCQM-K86 and P113.1 participants. (d) stands for dPCR, (*) laboratory participating to the study as a pilot study P113.1

7 CCQM-K86 and P113.1 participants' results

The CCQM-K86 and P113.1 participants results with the reported uncertainties, as reported to the coordinator, are given in **Tables 4** and **5**. The results are shown graphically in **Figures 1** and **2**.

Chi-squared test can be performed on the results to verify if some of the reported uncertainties were underestimated for the results on both materials.
The average of the study is given as an informative value in **Figures 1** and **2**.

Table 4: CCQM-K86 and P113.1 participants' measurement results for sample 1

	Reported results [x] MON810/*hmg* copy number ratio %	**Standard uncertainty [u] MON810/*hmg* copy number ratio %**	**Coverage factor [k]**	**Expanded uncertainty [U] cp/cp %**	**Relative uncertainty [u/x] %**
AIST	0.27	0.023	2.00	0.046	8.52
CENAM	0.52	0.09	2.00	0.18	17.31
DMSc	0.58	0.12	2.00	0.24	20.69
GLHK	0.50	0.085	2.00	0.17	17.00
IRMM	0.45	0.04	2.00	0.08	5.56
IRMM (d)	0.49	0.025	2.00	0.04	5.13
KRISS	0.542	0.0344	2.57	0.0884	6.35
Lab 1 (d)*	0.27	Approx. 0.02	2.16	-0.04/+0.04	Approx. 7.4
Lab 2.1*[1]	0.3974	0.0312	2.00	0.0624	7.81
Lab 2.2*[2]	0.3487	0.0043	2.00	0.0086	1.23
Lab 3*	0.447	0.099	2.00	0.198	22.15
Lab 4*	0.47	0.025	2.00	0.05	5.32
LGC	0.48	0.055	2.00	0.11	11.46
MIRS/NIB	0.57	0.1	2.00	0.2	17.54
NIM[3]	0.37	0.04	2.00	0.08	10.81
NIST	0.50	0.075	2.00	0.15	15.00
NMIA (d)	0.441	0.022	2.00	0.045	5.00
TÜBİTAK UME	0.70	0.085	2.00	0.17	12.09
VNIIM	0.98	0.05	2.00	0.10	5.10

(d): determined by dPCR; (*): results submitted as P113.1; the affiliation of the Institute is not revealed; (cp): copies.

[1] JAS method / pMul5 calibrant [3]. This result was only reported by Lab 2 as information.
[2] EURL-GMFF method [2]/ERM-AD413 calibrant [1]
[3] NIM subsequently reported a new value for sample 1 of 0.42 cp/cp % with an expanded uncertainty of 0.07 based on 2 additional datasets that were not reported initially.

Table 5: CCQM-K86 and P113.1 participants' measurement results for sample 2

	Reported results [x] MON810/*hmg* copy number ratio %	Standard uncertainty [u] MON810/*hmg* copy number ratio %	Coverage factor [*k*]	Expanded uncertainty [U] cp/cp %	Relative uncertainty [u/x] %
AIST	1.5	0.075	2.00	0.15	5.00
CENAM	5.1	1.050	2.00	2.1	20.59
DMSc	2.48	0.520	2.00	1.04	20.97
GLHK	2.38	0.425	2.00	0.85	17.86
IRMM	1.89	0.100	2.00	0.20	5.29
IRMM (d)	2.26	0.15	2.00	0.30	6.64
KRISS	2.50	0.058	2.57	0.148	2.29
Lab 1 (d)*	1.81	Approx. 0.08	2.16	- 0.17/+0.19[4]	Approx. 4.4
Lab 2.1*[5]	1.935	0.048	2.00	0.0947	2.45
Lab 2.2*[6]	1.705	0.049	2.00	0.0974	2.86
Lab 3*	2.229	0.379	2.00	0.757	16.98
Lab 4*	2.10	0.240	2.00	0.48	11.43
LGC	2.23	0.23	2.00	0.46	10.31
MIRS/NIB	1.77	0.250	2.00	0.5	14.12
NIM[7]	1.53	0.090	2.00	0.18	5.88
NIST	2.24	0.325	2.00	0.65	14.51
NMIA (d)	2.018	0.091	2.00	0.18	4.5
TÜBİTAK UME	3.28	0.40	2.00	0.80	12.26
VNIIM	3.40	0.225	2.00	0.45	6.62

(d): determined by dPCR; (*): results submitted as P113.1; the affiliation of the Institute is not revealed.

[4] Reported as 95 % confidence interval ranging from 0.23 to 0.31 % GM

[5] JAS method/ pMul5 calibrant [3]. This result was only reported by Lab 2 as information.

[6] EURL method [2] /ERM-AD413 calibrant [1]

[7] NIM subsequently reported a new value for sample 2 of 1.81 cp/cp % with an expanded uncertainty of 0.19 based on 2 additional datasets that were not reported initially.

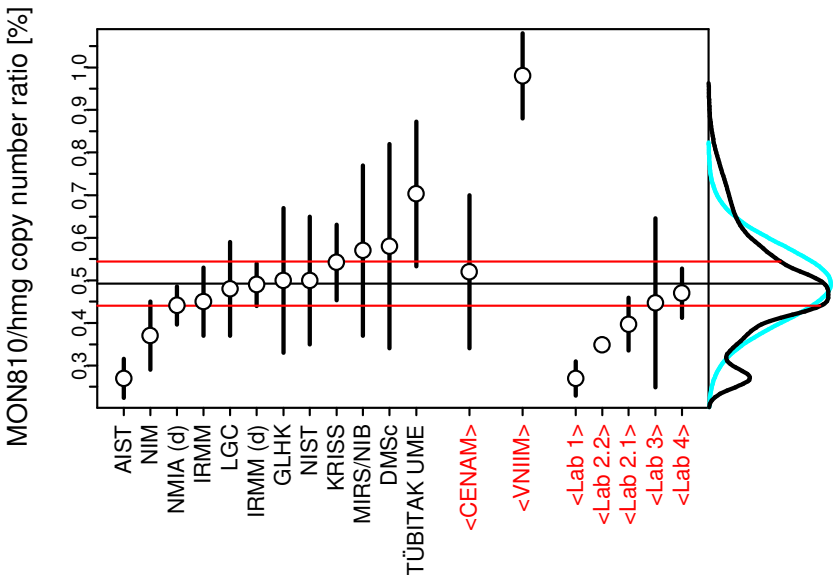

Figure 1: Reported results and uncertainties: sample 1
Horizontal lines represent the unweighted mean (black) with the 95 % confidence intervals (red); the black and blue lines to the right of the plot represent the Gaussian distribution and the dispersion estimate for the mean estimate, respectively. The results from laboratories in red were not used for the determination of the Key Comparison Reference Value (KCRV). Laboratories participating in the pilot study are marked as Lab.

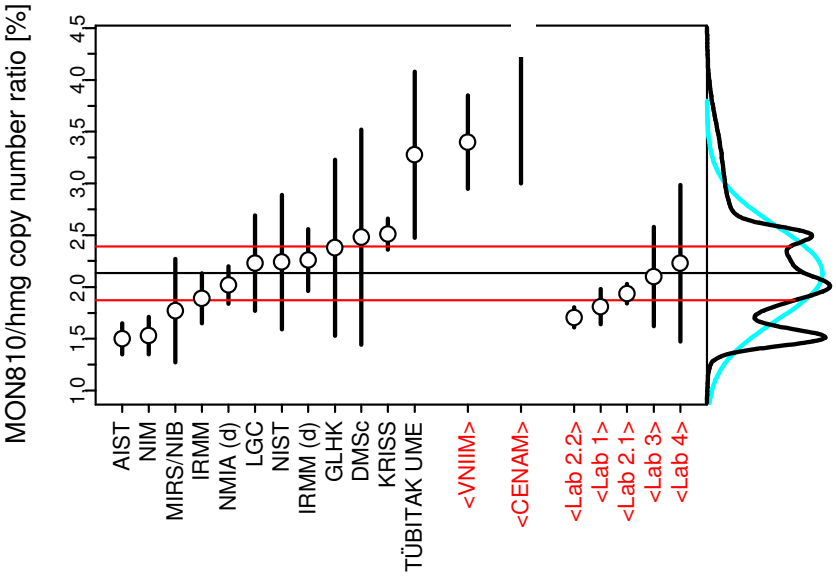

Figure 2: Reported results and uncertainties: sample 2
Horizontal lines represent the unweighted mean (black) with the 95 % confidence intervals (red); the black and blue lines to the right of the plot represent the Gaussian distribution and the dispersion estimate for the mean estimate, respectively. The results from laboratories in red were not used for the determination of the KCRV. Laboratories participating in the pilot study are marked as Lab.
The result from CENAM is not visible as Y-scale is limited to 4.5.

8 Key comparison reference value

The samples 1 and 2 contained different mass fractions of the GM MON810 maize that have been certified taking into account the combined standard uncertainties of the balances, of the water measurement uncertainties, of the inhomogeneity of the purity of the non-GM base material (based on the limit of detection (LOD) of the applied method, the purity of the GM base material, the number of seeds tested individually) and the uncertainty contribution for the long term stability estimated to be 1.1 % for 12 months (based on comparable maize materials). The assigned values were 8.1 ± 0.7 g/kg and 38.3 ± 1.7 g/kg (k = 2) for sample 1 and 2, respectively. The uncertainties related to the homogeneity and stability for both samples are provided in Annex A.

The key comparison reference value (KCRV) was estimated following the draft CCQM guidance note [4]. The qualified participants were those that participated to the CCQM-K86. The results from participants in the parallel pilot study P113.1 were not taken into account to determine the KCRV. The working group has defined the candidate set as the reported results calibrated for copy number ratio only. The results of one participant (CENAM) that had used a calibrant certified for the mass fraction was removed from the candidate set for the determination of the KCRV. The calibrant used by CENAM was a series of candidate CRMs produced by CENAM and certified for its MON810 mass fraction. The copy number ratio in those CENAM materials have been determined using ERM-AD413 calibrant in a subsequent study carried out by IRMM. That study indicated that the MON810 seeds used by CENAM were hemizygous seeds with paternal origin of the transgene. That difference could partially explain the high value of 5.1 % reported by CENAM for sample 2 as a conversion factor of 0.5 was used to convert mass fractions into copy number ratio whereas the ratio between MON810 and *hmg* copies in the calibrant used by CENAM was found to be rather around 0.37. CENAM explained that the result for sample 1 was in contrast only slightly overestimated as they noticed a stronger bias of their calibration curves towards higher concentrations. This example shows that the use of conversion factor to calculate copy numbers from mass certified material can generate errors.
Another participant (VNIIM) reported some technical problems related to the experimental setup. Upon request from VNIIM's participant, the working group agreed to not include results reported by VNIIM in the candidate set for the calculation of the KCRV. The amended results submitted by NIM after the key comparison dead line have not been taken into account for the calculation of the KCRV.

The screening of the data for consistency and anomalous values was performed by a preliminary inspection using a graphical method for samples 1 and 2. The measured values that deviate substantially relative to their reported uncertainties were identified by a plot of $[x_i\text{-med}(x)]/u(x_i)$ (Figures 3 and 4).

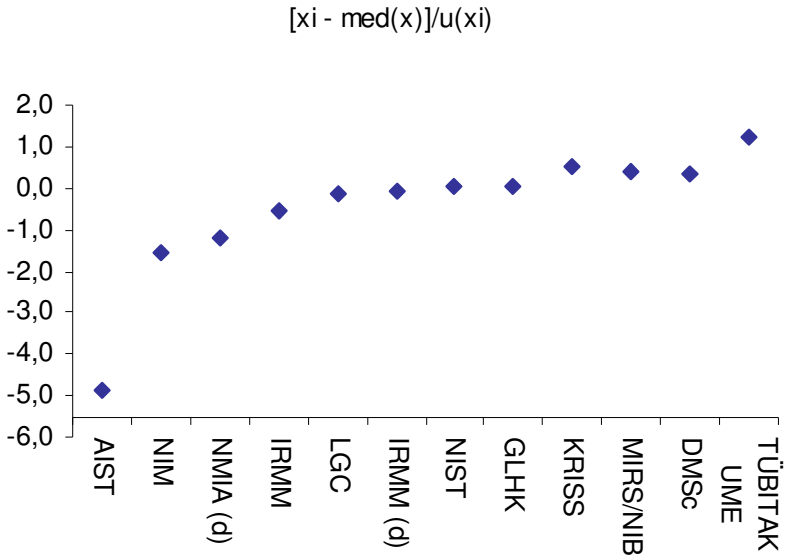

Figure 3: Identification of the results for sample 1 that are far from the median relative to their uncertainties.

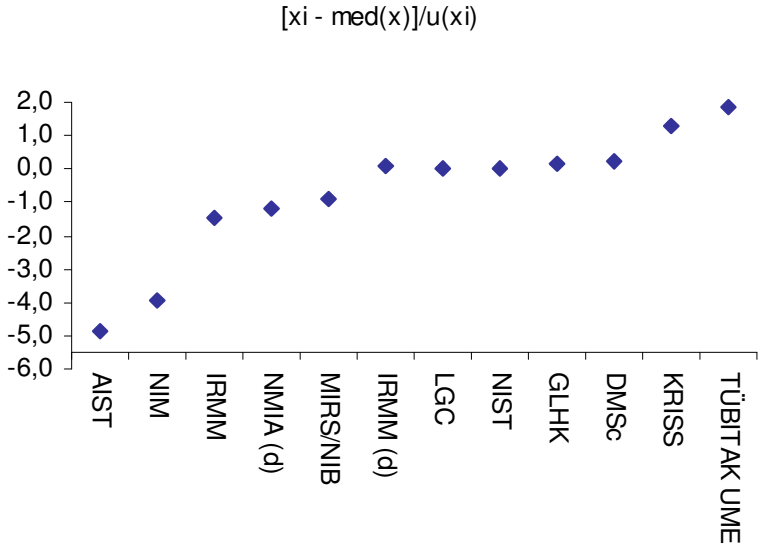

Figure 4: Identification of the results for sample 2 that are far from the median relative to their uncertainties.

The graphical inspection has been supported by outlier tests (Dixon's test, Nalimov t-test and Grubb's test) performed on the laboratories mean values. For sample 1, AIST and TÜBİTAK UME reported mean values of 0.27 and 0.70, respectively, which were both indicated as outlier mean values in the Nalimov t-test at levels of significance α=0.05 with 10 degrees of freedom (n-2, where n is the number of laboratories). For sample 2, the mean value (3.28) reported by TÜBİTAK UME was indicated as an outlier value at levels of significance α=0.05 with 10 degrees of freedom. AIST had the lowest and TUBITAK UME the highest reported mean values for both samples 1 and 2, indicating a potential under- and over-estimation of the copy number ratio, respectively.

Extreme values were also identified by calculating a robust estimate of location $\hat{\mu}$ and dispersion $\hat{\sigma}$, and values considered as extreme when outside $\hat{\mu} \pm 2\hat{\sigma}$ (corresponding approximately 95 %

confidence). Using this approach, the same mean values indicated as outliers by the statistical tests were confirmed as extreme values.
Identified extreme or outlier means have not been removed from the candidate set to calculate the KCRV and preference was given to apply robust statistics.

Several location and dispersion estimates for samples 1 and 2 have been assessed using the PDF_MakerTotal exploratory consensus assessment tool developed by David Lee Duewer (NIST) (Figure 5 and Figure 6).

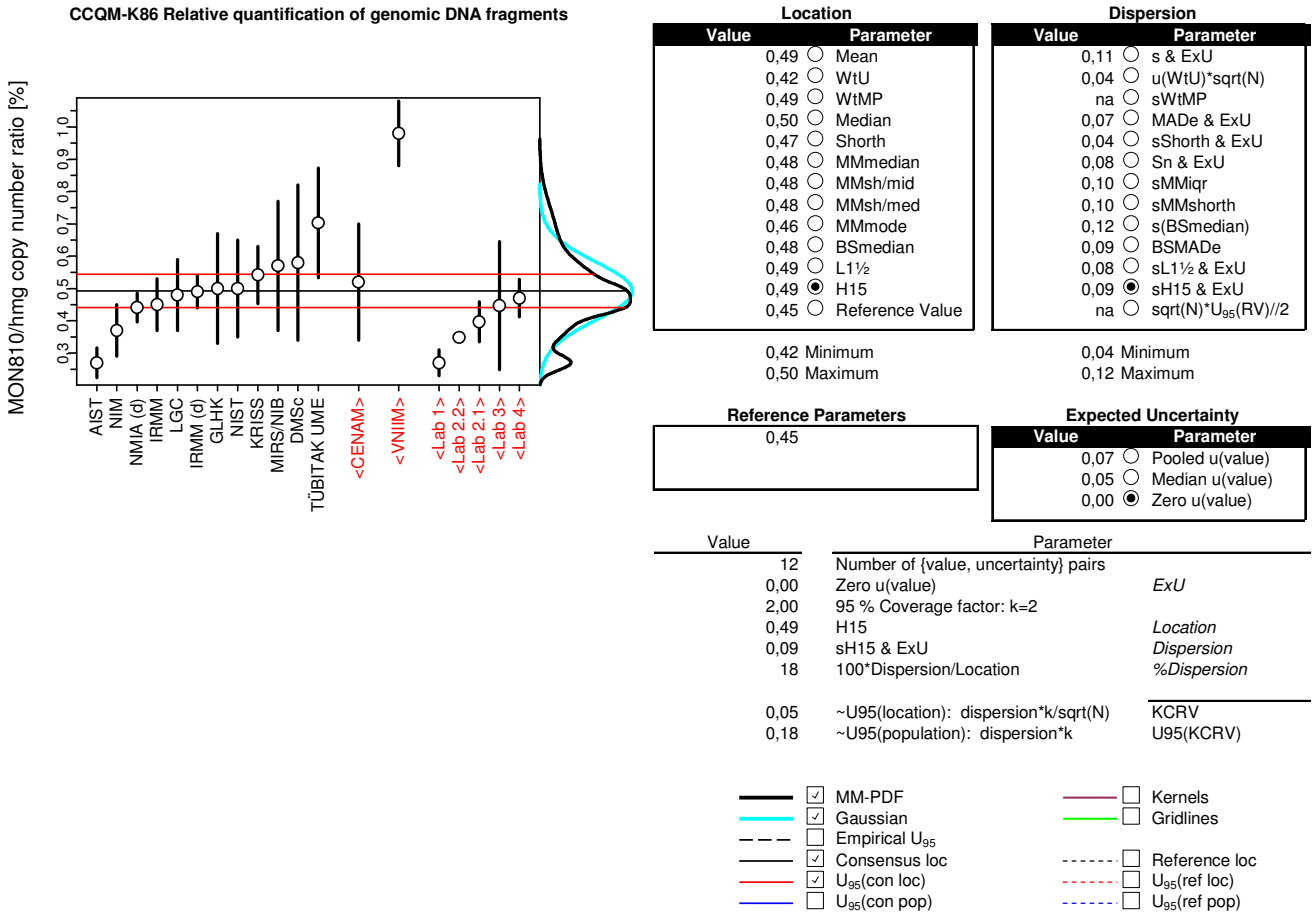

Figure 5: Location and dispersion of the estimates for sample 1. The Huber's estimate 2 winsorized mean [5] and dispersion is plotted here. The laboratories in red are displayed but not otherwise used for the determination of the KCRV. Laboratories participating as a pilot study are displayed with a red Lab number.

The estimated KCRV values for sample 1 vary from 0.42 to 0.50 depending on the estimator used with a dispersion value between 0.04 and 0.13. Those values are slightly higher than the consensus value of 0.45 obtained through collaborative studies on the same material using the same calibrant reference system (ERM-AD413) except for the Graybill-Deal weighted mean (WtU) [6] giving a 0.42 estimate.

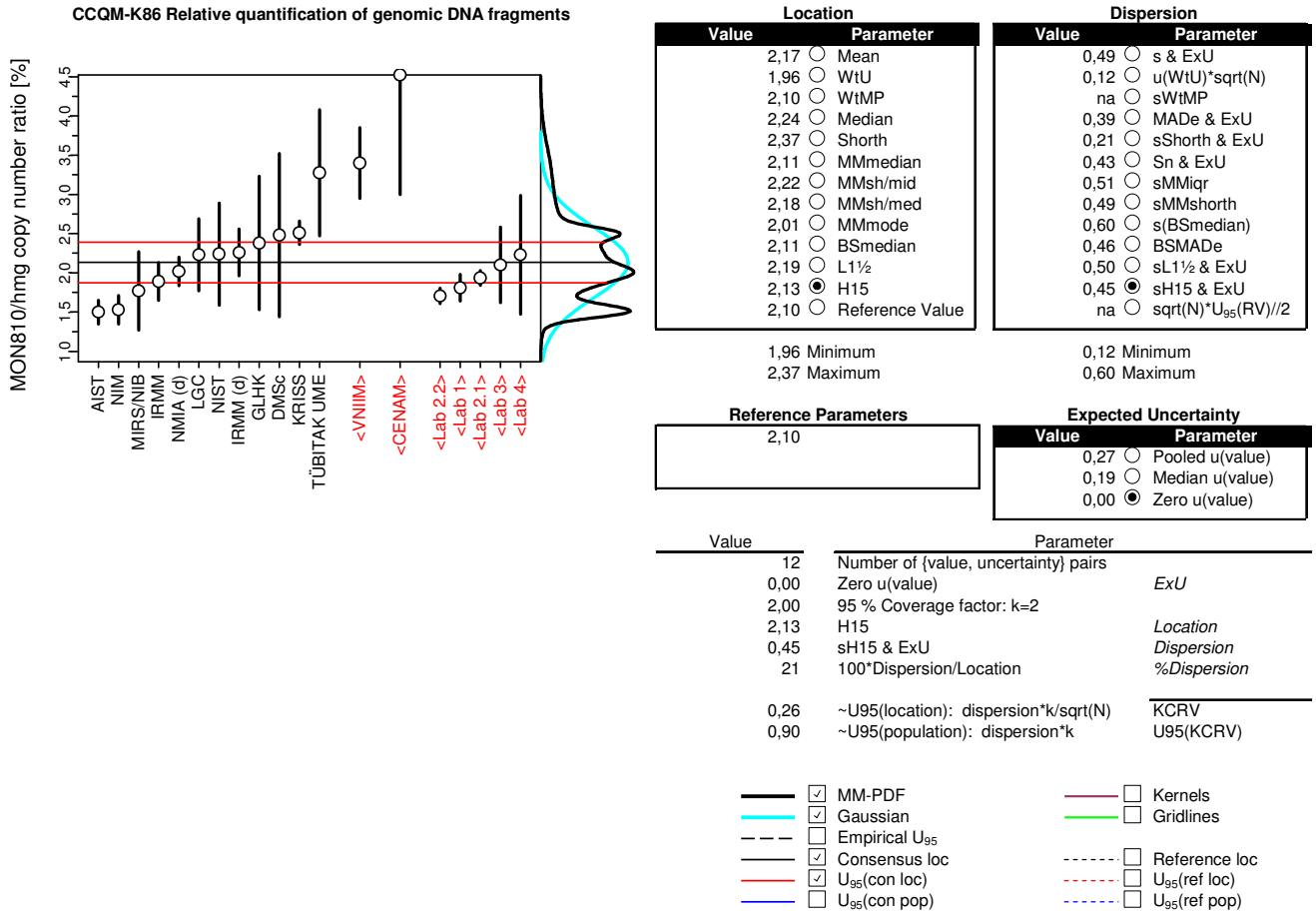

Figure 6: Location and dispersion of the estimates for sample 2. The Huber's estimate 2 winsorized mean and dispersion is plotted here. The laboratories in red are displayed but not otherwise used for the determination of the KCRV. Laboratories participating as a pilot study are displayed with a red Lab number.

The estimated KCRV values for sample 2 vary from 1.96 to 2.37 depending on the estimator used with a minimum and maximum dispersion value of 0.12 and 0.59, respectively. Those values are in agreement with the consensus value of 2.10 obtained trough collaborative studies on the same material using the same calibrant reference system (ERM-AD413). The dispersion of the data does not follow a smooth Gaussian shape again indicating some outlier values.

Huber's method was chosen for the calculation of the KCRV for samples 1 and 2 assuming that the underlying distribution is normal (almost unimodal and symmetrical) but contaminated with outliers. The Huber estimate 2 model was applied without regard to reported uncertainties (Table 7).

The standard uncertainty of the $\hat{\mu}_{H15}$ has been calculated using the formula $u^2(\hat{\mu}_{H15}) = \frac{1}{e}\hat{\sigma}^2_{H15}$, where $\hat{\sigma}^2_{H15}$ is the robust estimate of the standard deviation delivered simultaneously in the iterative estimation of $\hat{\mu}_{H15}$ and *e* is the efficiency (0.95 for *k* = 1.345).

Table 6: KCRV based on CCQM-K86

KCRV $\hat{\mu}_{H15}$	*MON810/hmg copy number ratio [%]*	*u [%]*	*U k=2 [%]*
Sample 1	0.49	0.03	0.06
Sample 2	2.13	0.13	0.26

The expanded uncertainty of the copy number ratio is calculated as being the robust estimate of the standard deviation divided by the square root of the number of dataset (laboratories) and multiplied by a coverage factor *k* of 2. The relative expanded uncertainties were identical for both samples and correspond to 13 % of the key comparison reference values.

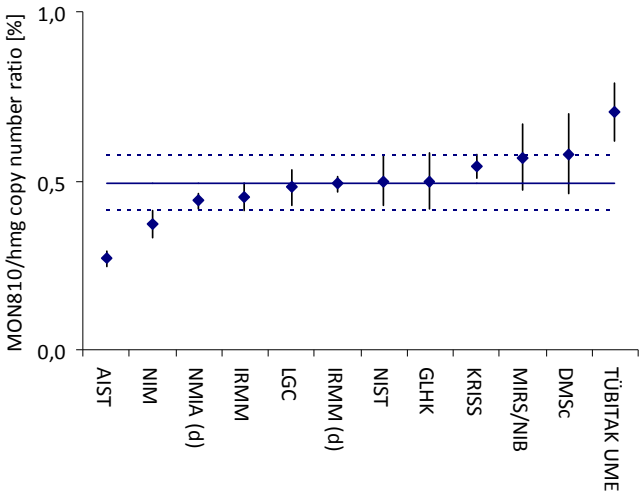

Figure 7: Reported results and standard uncertainties (*k* = 1) for sample 1. The dashed lines represent the expanded uncertainty of the KCRV, shown as a solid horizontal line.

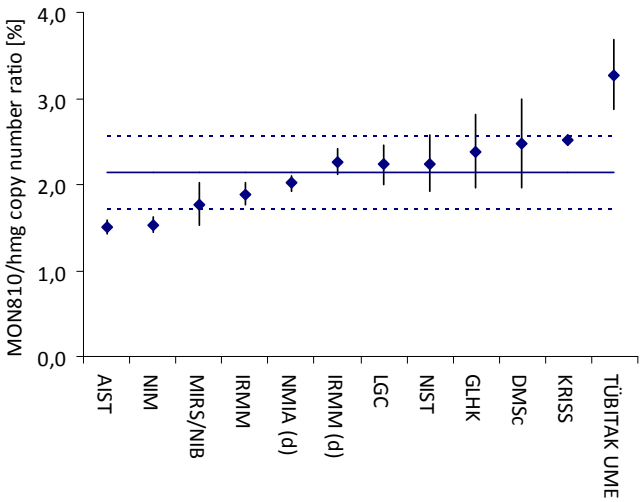

Figure 8: Reported results and standard uncertainties (*k* = 1) for sample 2. The dashed lines represent the expanded uncertainty of the KCRV, shown as a solid horizontal line.

9 Equivalence statements

9.1 Degree of equivalence with respect to KCRV

The equivalence statements are calculated following the CCQM guidance note [4], which specify that the degree of equivalence between a NMI result x_i and the KCRV $\hat{\mu}_{H15}$ is expressed as the difference D_i calculated as:

$$D_i = x_i - \hat{\mu}_{H15}$$

The uncertainty associated with the difference was expressed in the form of an expanded uncertainty. The uncertainty of the degree of equivalence (DoE) has been calculated as:

$$u^2(d_i) = \hat{\sigma}^2_{H15} + u^2(\hat{\mu}_{H15}) - 2\,\mathrm{cov}(x_i, \hat{\mu}_{H15})$$

The formula allows random effects that could increase the dispersion of the values x_i.
The degrees of equivalence calculated as above are shown in Table 7.

Degrees of equivalence (*rDi*) relative to the KCRV shown in Table 8 are calculated as:

$$rD_i = \frac{x_i - \hat{\mu}_{H15}}{\hat{\mu}_{H15}}$$

and illustrated graphically as relative degree of equivalence in Figures 9 and 10.

Table 7: Degrees of equivalence (D) with respect to KCRV

Sample 1			Sample 2		
Participant	*Di* [%]	U(*Di*) [%]	Participant	*Di* [%]	U(*Di*) [%]
AIST	-0.22	0.12	AIST	-0.63	0.56
NIM	-0.12	0.13	NIM	-0.60	0.57
NMIA (d)	-0.05	0.12	MIRS/NIB	-0.36	0.73
IRMM	-0.04	0.13	IRMM	-0.24	0.59
LGC	-0.01	0.15	NMIA (d)	-0.11	0.57
IRMM (d)	-0.002	0.12	IRMM (d)	0.13	0.61
NIST	0.008	0.18	LGC	0.10	0.71
GLHK	0.008	0.20	NIST	0.11	0.84
CENAM	0.03	0.21	GLHK	0.25	1.01
KRISS	0.05	0.14	DMSc	0.35	1.17
MIRS/NIB	0.08	0.23	KRISS	0.38	0.56
DMSc	0.09	0.26	TÜBİTAK UME	1.15	0.97
TÜBİTAK UME	0.21	0.20	VNIIM	1.27	0.70
VNIIM	0.49	0.15	CENAM	2.97	2.17

The unit of measurement is MON810/hmg copy number ratio expressed in percentage. The expanded uncertainty [(U(*Di*)] used a coverage factor *k* of 2.

Table 8: Relative degrees of equivalence (*rDi*) with respect to KCRV

Sample 1			Sample 2		
Participant	*rDi* [%]	U(*rDi*) [%]	Participant	*rDi* [%]	U(*rDi*) [%]
AIST	-45.2	23.6	AIST	-29.6	26.1
NIM	-24.8	27.1	NIM	-28.2	26.5
NMIA (d)	-10.4	23.5	MIRS/NIB	-16.9	34.4
IRMM	-8.6	27.1	IRMM	-11.3	27.6
LGC	-2.5	31.1	NMIA (d)	-5.3	26.5
IRMM (d)	-0.5	23.9	IRMM (d)	6.1	28.8
NIST	1.6	37.4	LGC	4.7	33.1
GLHK	1.6	40.7	NIST	5.2	39.5
CENAM	5.6	42.5	GLHK	11.7	47.2
KRISS	10.1	28.1	DMSc	16.4	54.9
MIRS/NIB	15.8	46.0	KRISS	17.8	26.1
DMSc	17.8	53.3	TÜBİTAK UME	53.8	45.3
TÜBİTAK UME	42.8	40.7	VNIIM	59.6	32.8
VNIIM	99.1	29.7	CENAM	139.4	101.7

The unit of measurement is relative MON810/hmg copy number ratio expressed in percentage. The expanded uncertainty [(U(*rDi*)] used a coverage factor *k* of 2.

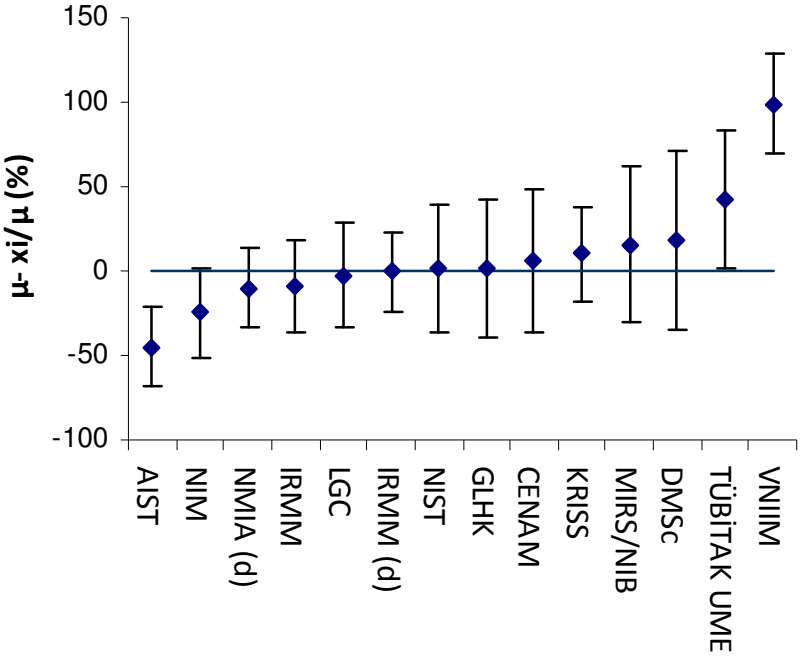

Figure 9: Relative degrees of equivalence with respect to KCRV – sample 1

The graph shows the relative degrees of equivalence between participant results for sample 1 and KRCV. Error bars show the uncertainties at *k*=2.

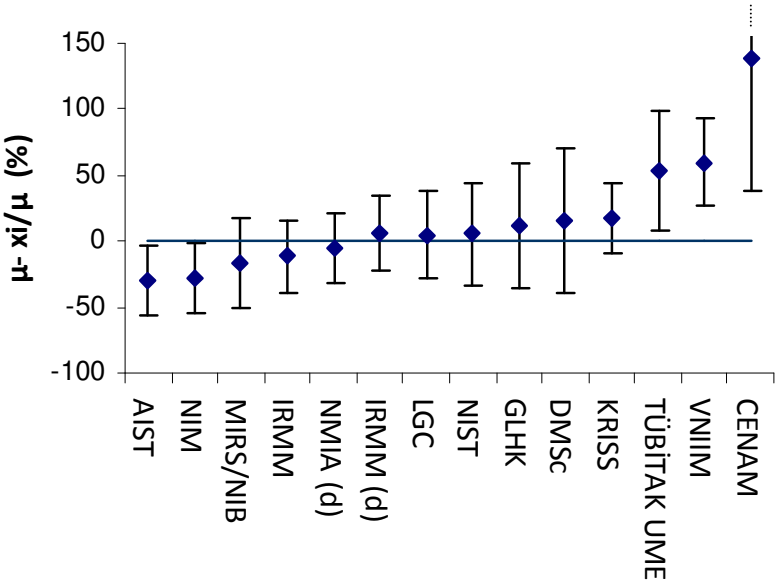

Figure 10: Relative degrees of equivalence with respect to KCRV – sample 2

The graph shows the relative degrees of equivalence between participant results for sample 2 and KRCV. Error bars show the uncertainties at *k*=2.

9.2 Degree of equivalence between laboratories

The pairwise degrees of equivalence between two laboratories *i* and *j* is given by the formula:

$$D_{i,j} = x_i - x_j$$

The uncertainty between two laboratories is given by:

$$u^2(d_{i,j}) = u_i^2 + u_j^2 - 2\mathrm{cov}(x_i, x_j)$$

The pairwise degrees of equivalence and uncertainties are shown in Appendix C.

10 Discussion

Good agreement was observed between the reported results of 11 participants. Several different extraction methods have been applied to extract the DNA from the maize tissues and led to very similar final results. This suggests that the DNA that has been extracted by the different methods was of similar quality and purity and could further be amplified during the PCR.

The relative quantification of the two genomic DNA fragments present in the ground maize seeds is traceable to the calibrant used, being plasmid DNA ERM-AD413 or genomic DNA extracted from the ERM-BF413d. Both calibrants were certified for their copy number ratio.

Some general aspects of quantification of GM material have not been addressed in CCQM-K86. Those concern mainly the sampling protocol, the ability to quantify DNA fragments presenting some degree of degradation (e.g. in highly processed food or feed) and the design of primers and probes that have not been verified in this study. The CCQM-K86 does not support the ability of either an NMI or a DI to to screen for the presence of unknown GM product in a biological tissue.

Within the K86, results obtained by qPCR were in agreement with the dPCR results (obtained without the use of an external calibrant). However as only 2 laboratories submitted results obtained by dPCR, more extensive studies should be organised to demonstrate that both methods deliver comparable results.

Finally, the results obtained after calibration with a certified pDNA calibrant (ERM-AD413) were very similar to those obtained using another plasmid calibrant (pMuI5) used in combination with another detection method (JAS) targeting other fragments of the MON810 genome. The expanded uncertainty obtained on samples 1 and 2 with the JAS method were larger but the reported values were close to the KCRV. In other words, this suggests that comparable results can be obtained using different plasmid calibrants and methods targeting different DNA fragments.

K86 should allowed NMIs and DIs to claim measurement capabilities for the relative quantification of genomic fragments in biological tissues taking into account the necessary mentioned limitations. The GM/non-GM ratios tested in K86 were relatively low and below the GM labelling thresholds currently

in place worldwide. The levels tested in this key comparison are therefore particularly relevant for GM analysis.

11 Acknowledgements

David L. Duewer (NIST) is strongly acknowledged for providing the exploratory consensus tool for interlaboratory data analysis (PDF_MakerTotal). Steve Ellison (LGC) is kindly acknowledged for his support in the data analysis.

12 References

[1] *http://irmm.jrc.europa.eu/catalogue*

[2] http://gmo-crl.jrc.ec.europa.eu/summaries/Mon810_validation_report.pdf

[3] Yoshimura T., Kuribara H., Matsuoka T., Kodama T., Iida M., Watanabe T., Akiyama H., Maitani T., Furui S., and Hino A. (2005) J. Agric. Food Chem. 53, 2052-2059.

[4] CCQM Guidance note: Estimation of a consensus KCRV and associated Degree of Equivalence. Version 6.2. Date 2010-03-01.

[5] Huber, P. J. (1964) Annals of Mathematical Statistics 35:73-101.

[6] Aiyappan Nair K. (1980) Variance and Distribution of the Graybill-Deal Estimator of the Common Mean of Two Normal Populations. The annals of statistics 212-216

[7] Charels D., Broeders S., Corbisier P., Trapmann S. Schimmel H., Emons H. (2007) J. Agric. Food Chem. 55, 3268-3274.

Appendix A: Sample preparation, homogeneity and stability studies

Different maize matrix powder GM materials from maize have been produced at IRMM during the past years and are undergoing regular long-term stability monitoring. It turned out that they possess identical stability properties.
Therefore, the uncertainty contribution for the long term stability could be taken from a previous certification study (relative uncertainty contribution 1.1 % for a time interval of 12 months and GM fractions between 0.8 % and 4.5 % m/m).

A further contribution to the combined uncertainty comes from the homogeneity assessment. Its relative value was calculated to be 3.4 % and 1.7 % for the samples 1 and 2, respectively.

The expanded combined standard uncertainty of the certified value has also been determined for samples 1 and 2. The uncertainty, which can be expected for certified values of copy number ratios in matrix RMs, was calculated on the basis of results obtained by previous studies performed at IRMM. The average copy number ratios and their uncertainties were calculated from the data of the event-specific detection method calibrated with ERM-AD413 and using different DNA extraction methods (Table 9).

The uncertainty contribution from the characterization was determined on the basis of an interlaboratory study with 43 participating laboratories having performed qPCR using ERM-AD413 as a calibrant on the same materials.

Table 9: Average copy number ratios and expanded combined uncertainties (U_{CRM}. $k = 2$) for the event-specific detection method, obtained with ERM-AD413 as calibrant.
U_{rel} refers to the relative expanded combined uncertainty; cp no. copy number.

	Relative standard uncertainty contribution				
Mass fraction [%]	Average cp no ratio	Homogeneity[1] [%]	Stability[2] [%]	Characterization[3] [%]	U_{rel} [%]	U_{CRM} [cp no ratio]
0.8	0.45	3.4	1.1	3.9	11	0.05
3.8	2.1	1.7	1.1	3.4	8	0.17

[1] The uncertainty contribution from the homogeneity is relative to a mass fraction.
[2] This uncertainty contribution was estimated using data from previous long term stability studies and is expressed relative to a mass fraction.
[3] The uncertainty contribution was assessed using data from a previous study and is expressed relative to a copy number ratio [7].

Appendix B: Shipping document distributed to participants

Shipping document

Geel, 1st June 2010
Ref: CCQM-K86 – P113.1

Dear participant to the KC86/P113.1.

Enclosed are the 2 samples to be analysed in the CCQM-K86 – P113.1 entitled "Relative quantification of genomic DNA fragments extracted from a biological tissue".

You should store the samples at + 4°C upon arrival.

Please make sure to read carefully through all information related to the KC that has been sent by e-mail.

A total of 13 NMIs have signed in for the KC and 3 laboratories as a pilot study.

We sincerely wish to thank you once again for your willingness to participate in this KC86 – P113.1 and we wish you good luck in the analysis. Your data should be sent to me **before the 6th of October 2010.** This will allow me to present a draft report at the next BAWG WG in November 2010.

Please confirm the receipt of the materials.

Kind regards.

Dr Philippe Corbisier
Mrs Anne-Marie Kortekaas
Retieseweg 111. B-2440 Geel. Belgium
tel : +3214 571890 (Office)
fax: +3214 571548
e-mail : philippe.corbisier@ec.europa.eu
web : http://www.irmm.jrc.be
CRMs : http://irmm.jrc.europa.eu/catalogue
50 years : http://irmm.jrc.ec.europa.eu/50.

A detailed questionnaire was submitted to the participants. The participants shall submit an electronic version of the reporting template and questionnaire by e-mail to philippe.corbisier@ec.europa.eu. 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 philippe.corbisier@ec.europa.eu. The participants shall burn a copy of the report and of the raw data on a CD-ROM. Raw data of the preparation of the calibration curves and unknowns should be provided.

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 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 or fax to:

Dr Philippe Corbisier
European Commission
IRMM
Retieseweg 111
2440 Geel
BELGIUM
Phone: +32 (0)14 571 890
Fax: +32 (0)14 571 548
E-mail: philippe.corbisier@ec.europa.eu

Appendix C: Pairwise equivalence tables

The following tables show degree of equivalence between participant laboratories in CCQM-K86. The values are given in MON810 cp/*hmg* cp number ratio expressed in percentage.

CCQM-K86: Sample 1

	AIST	NIM	NMIA (d)	IRMM	LGC	IRMM (d)	NIST	GLHK	CENAM	KRISS	MIRS/NIB	DMSc	TÜBİTAL-UME	VNIIM
AIST	-	-0.10	-0.17	-0.18	-0.21	-0.22	-0.23	-0.23	-0.25	-0.27	-0.30	-0.31	-0.43	-0.71
NIM	0.10	-	-0.07	-0.08	-0.11	-0.12	-0.13	-0.13	-0.15	-0.17	-0.20	-0.21	-0.33	-0.61
NMIA (d)	0.17	0.07	-	-0.01	-0.04	-0.05	-0.06	-0.06	-0.08	-0.10	-0.13	-0.14	-0.26	-0.54
IRMM	0.18	0.08	0.01	-	-0.03	-0.04	-0.05	-0.05	-0.07	-0.09	-0.12	-0.13	-0.25	-0.53
LGC	0.21	0.11	0.04	0.03	-	-0.01	-0.02	-0.02	-0.04	-0.06	-0.09	-0.10	-0.22	-0.50
IRMM (d)	0.22	0.12	0.05	0.04	0.01	-	-0.01	-0.01	-0.03	-0.05	-0.08	-0.09	-0.21	-0.49
NIST	0.23	0.13	0.06	0.05	0.02	0.01	-	0.00	-0.02	-0.04	-0.07	-0.08	-0.20	-0.48
GLHK	0.23	0.13	0.06	0.05	0.02	0.01	0.00	-	-0.02	-0.04	-0.07	-0.08	-0.20	-0.48
CENAM	0.25	0.15	0.08	0.07	0.04	0.03	0.02	0.02	-	-0.02	-0.05	-0.06	-0.18	-0.46
KRISS	0.27	0.17	0.10	0.09	0.06	0.05	0.04	0.04	-0.02	-	-0.03	-0.04	-0.16	-0.44
MIRS/NIB	0.30	0.20	0.13	0.12	0.09	0.08	0.07	0.07	-0.05	0.03	-	-0.01	-0.13	-0.41
DMSc	0.31	0.21	0.14	0.13	0.10	0.09	0.08	0.08	-0.06	0.04	0.01	-	-0.12	-0.40
TÜBİTAL-UME	0.43	0.33	0.26	0.25	0.22	0.21	0.20	0.20	-0.18	0.16	0.13	0.12	-	-0.28
VNIIM	0.71	0.61	0.54	0.53	0.50	0.49	0.48	0.48	-0.46	0.44	0.41	0.40	-0.28	-

CCQM-K86: Sample 2

	AIST	NIM	MIRS/NIB	IRMM	NMIA (d)	IRMM (d)	LGC	NIST	GLHK	DMSc	KRISS	TÜBİTAL-UME	VNIIM	CENAM
AIST	-	-0.03	-0.27	-0.39	-0.52	-0.76	-0.73	-0.74	-0.88	-0.98	-1.01	-1.78	-1.90	-3.60
NIM	0.03	-	-0.24	-0.36	-0.49	-0.73	-0.70	-0.71	-0.85	-0.95	-0.98	-1.75	-1.87	-3.57
MIRS/NIB	0.27	0.24	-	-0.12	-0.25	-0.49	-0.46	-0.47	-0.61	-0.71	-0.74	-1.51	-1.63	-3.33
IRMM	0.39	0.36	0.12	-	-0.13	-0.37	-0.34	-0.35	-0.49	-0.59	-0.62	-1.39	-1.51	-3.21
NMIA (d)	0.52	0.49	0.25	0.13	-	-0.24	-0.21	-0.22	-0.36	-0.46	-0.49	-1.26	-1.38	-3.08
IRMM (d)	0.76	0.73	0.49	0.37	0.24	-	-0.03	-0.02	-0.12	-0.22	-0.25	-1.02	-1.14	-2.84
LGC	0.73	0.70	0.46	0.34	0.21	0.03	-	-0.01	-0.15	-0.25	-0.28	-1.05	-1.17	-2.87
NIST	0.74	0.71	0.47	0.35	0.22	0.02	0.01	-	-0.14	-0.24	-0.27	-1.04	-1.16	-2.86
GLHK	0.88	0.85	0.61	0.49	0.36	0.12	0.15	0.14	-	-0.10	-0.13	-0.90	-1.02	-2.72
DMSc	0.98	0.95	0.71	0.59	0.46	0.22	0.25	0.24	0.10	-	-0.03	-0.80	-0.92	-2.62
KRISS	1.01	0.98	0.74	0.62	0.49	0.25	0.28	0.27	0.13	0.03	-	-0.77	-0.89	-2.59
TÜBİTAL-UME	1.78	1.75	1.51	1.39	1.26	1.02	1.05	1.04	0.90	0.80	0.77	-	-0.12	-1.82
VNIIM	1.90	1.87	1.63	1.51	1.38	1.14	1.17	1.16	1.02	0.92	0.89	-0.12	-	-1.70
CENAM	3.60	3.57	3.33	3.21	3.08	2.84	2.87	2.86	2.72	2.62	2.59	-1.82	-1.70	-

The following tables show uncertainties between participant laboratories in CCQM-K86. Expanded uncertainties *U(Dij)* are given using a coverage factor *k=2* throughout. The values are given in MON810 cp/*hmg* cp number ratio expressed in percentage.

CCQM-K86: Sample 1

	AIST	NIM	NMIA (d)	IRMM	LGC	IRMM (d)	NIST	GLHK	KRISS	CENAM	MIRS/NIB	DMSc	TÜBİTAL-UME	VNIIM
AIST	-	0.09	0.09	0.09	0.12	0.07	0.16	0.18	0.10	0.19	0.21	0.24	0.18	0.11
NIM	0.09	-	0.11	0.11	0.14	0.09	0.17	0.19	0.12	0.20	0.22	0.25	0.19	0.13
NMIA (d)	0.06	0.09	-	0.09	0.12	0.07	0.16	0.18	0.10	0.19	0.21	0.24	0.18	0.11
IRMM	0.09	0.11	0.11	-	0.14	0.09	0.17	0.19	0.12	0.20	0.22	0.25	0.19	0.13
LGC	0.12	0.14	0.14	0.14	-	0.12	0.19	0.20	0.14	0.21	0.23	0.26	0.20	0.15
IRMM (d)	0.07	0.09	0.09	0.09	0.12	-	0.16	0.18	0.10	0.19	0.21	0.25	0.18	0.11
NIST	0.16	0.17	0.17	0.17	0.19	0.16	-	0.23	0.17	0.23	0.25	0.28	0.23	0.18
GLHK	0.18	0.19	0.19	0.19	0.20	0.18	0.23	-	0.19	0.25	0.26	0.29	0.24	0.20
CENAM	0.19	0.20	0.20	0.20	0.21	0.19	0.23	0.25	-	0.25				0.21
KRISS	0.10	0.12	0.12	0.12	0.14	0.10	0.17	0.19	0.13	-	0.22	0.26	0.19	0.13
MIRS/NIB	0.21	0.22	0.22	0.22	0.23	0.21	0.25	0.26	0.22	0.27	-	0.31	0.26	0.22
DMSc	0.24	0.25	0.25	0.25	0.26	0.25	0.28	0.29	0.26	0.30	0.31	-	0.29	0.26
TÜBİTAL-UME	0.18	0.18	0.19	0.19	0.20	0.18	0.23	0.24	0.19	0.25	0.26	0.29	-	0.20
VNIIM	0.11	0.11	0.13	0.13	0.15	0.11	0.18	0.20	0.13	0.21	0.22	0.26	0.20	-

CCQM-K86: Sample 2

	AIST	NIM	MIRS/NIB	IRMM	NMIA (d)	IRMM (d)	LGC	NIST	GLHK	DMSc	KRISS	TÜBİTAL-UME	VNIIM	CENAM
AIST	-	0.23	0.52	0.28	0.23	0.34	0.48	0.67	0.86	1.05	0.21	0.82	0.47	2.11
NIM	0.23	-	0.53	0.30	0.25	0.35	0.49	0.67	0.87	1.06	0.23	0.82	0.48	2.11
MIRS/NIB	0.52	0.53	-	0.55	0.53	0.58	0.68	0.82	0.99	1.15	0.52	0.95	0.67	2.16
IRMM	0.28	0.30	0.55	-	0.30	0.38	0.52	0.69	0.88	1.07	0.28	0.84	0.51	2.11
NMIA (d)	0.23	0.25	0.53	0.30	-	0.35	0.49	0.67	0.87	1.06	0.23	0.82	0.48	2.11
IRMM (d)	0.34	0.35	0.58	0.38	0.35	-	0.55	0.72	0.90	1.08	0.33	0.86	0.54	2.12
LGC	0.48	0.49	0.68	0.52	0.49	0.55	-	0.80	0.97	1.14	0.48	0.93	0.64	2.15
NIST	0.67	0.67	0.82	0.69	0.67	0.72	0.80	-	1.07	1.23	0.67	1.03	0.79	2.20
GLHK	0.86	0.87	0.99	0.88	0.87	0.90	0.97	1.07	-	1.34	0.86	1.17	0.96	2.27
DMSc	1.05	1.06	1.15	1.07	1.06	1.08	1.14	1.23	1.34	-	1.05	1.31	1.13	2.34
KRISS	0.21	0.23	0.52	0.28	0.23	0.33	0.48	0.67	0.86	1.05	-	0.82	0.47	2.11
TÜBİTAL-UME	0.82	0.82	0.95	0.84	0.82	0.86	0.93	1.03	1.17	1.31	0.82	-	0.92	2.25
VNIIM	0.47	0.48	0.67	0.51	0.48	0.54	0.64	0.79	0.96	1.13	0.47	0.92	-	2.15
CENAM	2.11	2.11	2.16	2.11	2.11	2.12	2.15	2.20	2.27	2.34	2.11	2.25	2.15	-

Appendix D: Information sent to the participants

Two maize powders each containing a defined mass fraction of genetically modified (GM) MON810 maize. The samples were produced under the responsibility of the IRMM and were prepared by mixing of dried non-GM maize powder and MON810 GM dried maize powder.

For specific detection of event MON810 maize a 92 bp fragment of the single copy DNA integration-border region of the genomic sequence and the inserted sequence element originating from CaMV (35S promoter) as a result of in vitro recombination present in the GM insect-protected MON810 ("YieldGuard") maize (Monsanto) is amplified in TaqMan® PCR.
For relative quantification of MON810 maize, a 79 bp fragment of the taxon specific maize (*Zea mays*) high mobility group protein gene (*hmg*) gene using a gene specific combination of primers and probe is amplified.

For the real-time PCR quantification a calibrant such as the ERM-AD413 can be used. The ERM-AD413 was processed and certified according to the ISO Guide 34 and is available for sale[11]. The CRM is certified for the number of DNA fragments per plasmid of a MON810 transgenic sequence and of the high mobility group gene (*hmg*). ERM-AD413 is intended to be used for the calibration of MON810 maize QRT-PCR method as described and validated by the Community Reference Laboratory for GM Food and Feed[12].

The ratio between the copy number of those two DNA sequences in the sample 1 and 2 must be determined:

Target sequence 1: (92 bp)
gCCACCTTCCTTTTCCACTATCTTCACAATAAAgTgACAgATAgCTgggCAATggCAAAggATgTTAAACgTTAgAgTCCT
TCgTCCTTCgA

Target sequence 2: (79 bp)
GCTACATAgggAgCCTTgTCCTACAATCCACACAAACgCACgCgTAAAACAATTAATCAgCACgAgATTTCTAgTCCAA

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 MON810 and *hmg* fragments measured.

Participants will also have the possibility to measure the absolute number of both DNA targets by digital PCR and to provide the ratio of those two numbers.

Homogeneity testing

Homogeneity analysis of the DNA sequences in sample 1 and 2 has been performed by IRMM and the uncertainty related to the homogeneity will be provided. The sample intake used for determining the homogeneity was 200 mg.

[11] http://irmm.jrc.europa.eu/catalogue
[12] http://gmo-crl.jrc.ec.europa.eu/summaries/Mon810_validation_report.pdf

Stability testing

Because samples 1 and 2 are similar to the ERM-BF413d CRM (seed powder kept under argon atmosphere) produced by IRMM which was proven to have sufficient short- and long-term stability, stability testing of samples 1 and 2 was deemed unnecessary.

Instructions for use

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

Methodology

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 CRL for GM Food and Feed can be applied[13].
Real-time PCR is the most commonly used method to quantify DNA sequences but other technologies can also be used. The PCR platform and chemistry can be chosen by the participants.

Timetable

Deadline for signup to the study[14]:	7th May 2010
Distribution of sample materials:	week of 6th June 2010
Deadline for submission of results:	6th October 2010
Draft report:	BAWG meeting Nov 2010
Circulation of draft final report:	BAWG meeting April 2011

[13] http://gmo-crl.jrc.ec.europa.eu/summaries/Mon810_validation_report.pdf

[14] 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.