# Final report for CCQM-K86.b Relative quantification of Bt63 in GM rice matrix sample

**Contact point**

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**Abstract**

Key comparison CCQM-K86.b was performed to demonstrate and document 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 rice powder. 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 up to 150 nucleotides following extraction from an unprocessed, high starch ground seed matrix, with a copy number ratio from 0.005 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 Institute of Metrology, China (NIMC) and Government Laboratory Hong Kong (GLHK).The following laboratories (in alphabetical order) participated in this key comparison study: CENAM (Mexico); GLHK (Hong Kong); LGC (United Kingdom); NIB (Slovenia); NIMC (P.R. of China); NIMT (Thailand); NMIA (Australia); NMIJ (Japan); TÜBITAK-UME (Turkey). Good agreement was observed between the reported results.

## 1. Introduction

In the past two decades, there has been a vast increase in the global plant area of genetically modified (GM) crops. The commercial planting of GM crops contributes to global feed, fiber, food, fuel as well as environment due to reduction of pesticide application; however, the safety of GM crops and its products have been prompted as an important issue. To facilitate international trade and to provide information to consumers, labeling requirements have been set up in many countries. Rice is an important grain in rice-growing nations and is consumed in large quantities. In order to improve the yield of rice, some GM varieties including pest resistance, bacterial resistance and herbicide tolerance of rice are being developed all over the world. Bt63 rice is genetically modified pest-resistant rice developed by Huazhong Agriculture University in China. Field-testing showed that Bt63 rice could reduce pesticide application and increase rice production through resistance against yellow stem borers and leaf-folders [1].

In 2009, the safety permission certificate issued by Chinese Ministry of Agriculture was granted for Bt63 (Shanyou 63, TT51-1) rice following the food and environment assessment. Bt63rice is the first GM food crop to be granted a safety certificate in China. Before Bt63 was approved for commercial planting, contamination of exported rice with Bt63 was found and reported by the European Commission, which implemented emergency measures requiring monitoring for Bt63 in rice. In order to protect the rice importers in countries in which Bt63rice is not authorized, the monitoring of Bt63 rice should be strengthened. Therefore, reference materials for Bt63 rice are needed to ensure that tests are accurate, reliable, and

comparable.

This study (CCQM-K86.b) aims to extend CMC claim supported by CCQM-K86 [2], to include a high starch matrix ((75-80 g/100g) in polymeric carbohydrate (amylose and amylopectin)). In this study, Bt63 rice and wild type (wt) rice were mixed in different ratios and the copy number ratio of event-specific sequence and endogenous gene in the genomic DNA extracted from the Bt63 rice matrix were to be determined by participants.

The quantification was performed by real-time quantitative Polymerase Chain Reaction (qPCR) and digital PCR (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.b and a pilot study, CCQM-P113.3, based on materials provided by NIMC.

## 2. Measurement Claim

The measurement claim for CCQM-K86.b 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 starch ground seed matrix, with a copy number ratio from 0.005 to 1**".The lower limit for fragment length depends on the particular primer set. A capability for measurement of a copy number ratio of 0.005 indicates a capability to measure a copy number ratio of 200 (1/0.005).

The study supports the participants' competence to extract DNA from a high starch matrix and to perform measurements on the extracted DNA using quantitative real-time PCR (“qPCR”) where an independent reference material is used as calibrant. The study also supports the competence of participants who have determined absolute amounts of DNA targets using digital PCR (“dPCR”) measurements.

The matrix is defined as a high starch plant origin, composed of ground seeds material requiring an optimized DNA extraction method. The measurand is the copy number ratio between the Bt63 inserted sequence and the endogenous gene.

Participants were asked to report the ratio as a percentage, that is, 100*$n$(Bt63)/($n$(reference). Both DNA fragments were present in a powder made by gravimetrically mixing of genetically modified rice (Bt63 or TT51-1 event) into non-genetically modified rice.

The dissemination range of measurement capability goes from 0.5 % to 3.5 % (copy number ratio expressed in %). The materials tested were assigned a mass fraction based on the gravimetric dry-mixing of non-modified rice powder with Bt63 rice powder. The assigned values were respectively 10.0 ± 0.1 g/kg and 35.0±1.1 g/kg ($k$=2) for Sample 1 and 2.

The determination of the ratio by qPCR was realized by using a plasmid calibrant (GBW10090) certified for its copy number ratio. The GBW10090 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 CCQM-K86.b did not support the design of primers and probe as this information was provided to the participants. The samples used in the CCQM-K86.bwere unprocessed samples from which high molecular weight genomic DNA could be extracted. The current K86.b can therefore not be used to claim 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 Bt63 but based on qPCR can nevertheless be supported by this Key Comparison (KC) provided that an appropriate calibrant is available for the qPCR procedure applied.

## 3. Participation in CCQM-K86.b

The nine NMIs / DIs that participated in CCQM-K86.b 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.b participants.**

| Institute / Organisation | Country | Contact |
|---|---|---|
| NIMC | China | L. Dong |
| GLHK | China | F.W. Lee |
| CENAM | Mexico | M. P. Urquiza |
| LGC | United Kingdom | M. Burns |
| NIB | Slovenia | M. Milavec |
| NIMT | Thailand | C. Prawettongsopon |
| NMIA | Australia | K. Griffiths |
| NMIJ | Japan | S. Shibayama |
| TUBITAK UME | Turkey | M. Akgöz |

## 4. Study Materials

### 4.1 Test sample

Two samples of rice powder each containing a defined mass fraction of genetically modified Bt63 rice were prepared as the materials for this study. The samples were produced under the responsibility of the NIM and were prepared by mixing of dried non-GM rice powder and dried Bt63 GM rice powder at different mass fractions.

### 4.2 Calibrant

A calibrant (CRM-pNIM-003) (GBW10090) for the real-time PCR quantification (qPCR) was provided by NIMC. The pNIM-003 was prepared according to the ISO Guide 34. The CRM is certified for the number of DNA fragments per plasmid of a Bt63 transgenic sequence and of the endogenous gene (starch branching enzyme gene, *RBE4*) as 1:1. This CRM (GBW10090) is intended to be used for the calibration of Bt63 rice relative quantification with the qPCR method.

### 4.3Homogeneity testing

From the batch of 200 vials of each test sample, 11 vials were randomly chosen for homogeneity testing. The results of quantification by droplet digital PCR (ddPCR) arepresented in Figure 1. The results of an F-test were $F$=1.21 and $F$=0.79 for sample 1 and sample 2, respectively ($p$ = 0.34 and $p$ = 0.64 respectively, based on 10 and 22 degrees of freedom for the between- and within-group terms). Therefore, it was firmly concluded that test samples were homogeneous within the measurement uncertainty. The sample intake used for determining the homogeneity was 100 mg. The relative uncertainty associated with inhomogeneity was calculated to be 1.4 % and 2.0 % for sample 1 and sample 2, respectively.

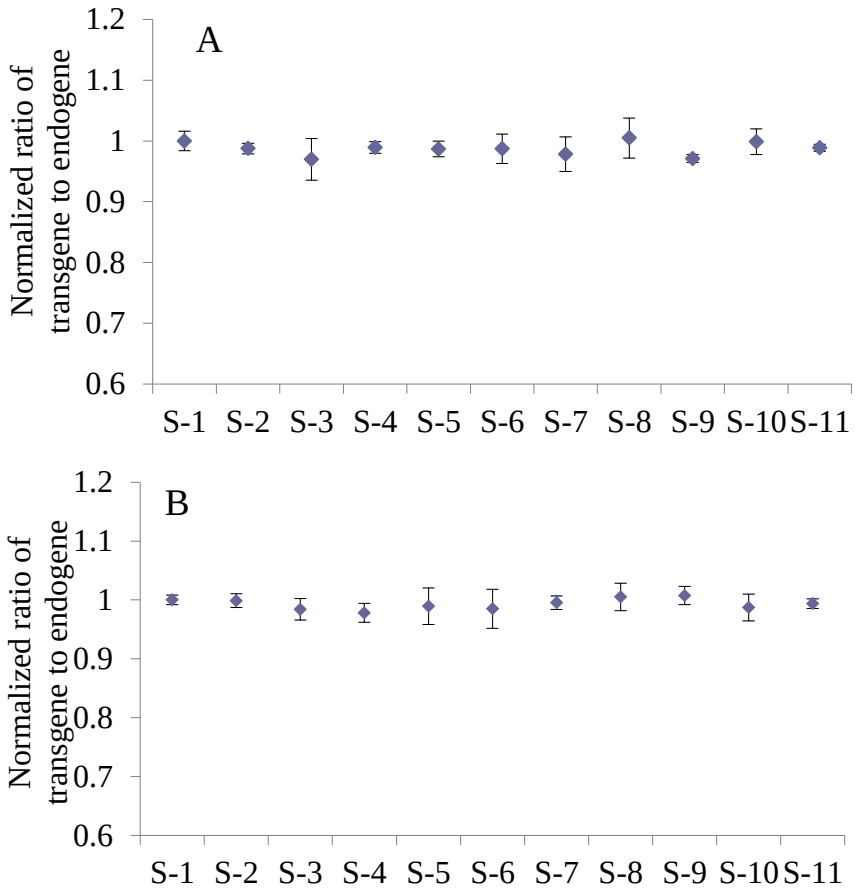


**Figure 1. The result of homogeneity test.**

(A) Sample 1; (B) Sample 2. Each sample vial was measured 3 times where 11 vials were measured simultaneously in the exactly same manner.

### 4.4 Stability testing

Short term stability of the test samples to reflect conditions during international delivery was examined by measuring samples at -70 ºC, 4 ºC, 25 ºC and 45 ºC. Measurement was performed by ddPCR, of which results are presented in Figure 2. Both *t*-test and trend analysis showed that copies of endogene and transgene in sample 1 and 2 are stable at all the tested temperatures tested for 4 weeks.

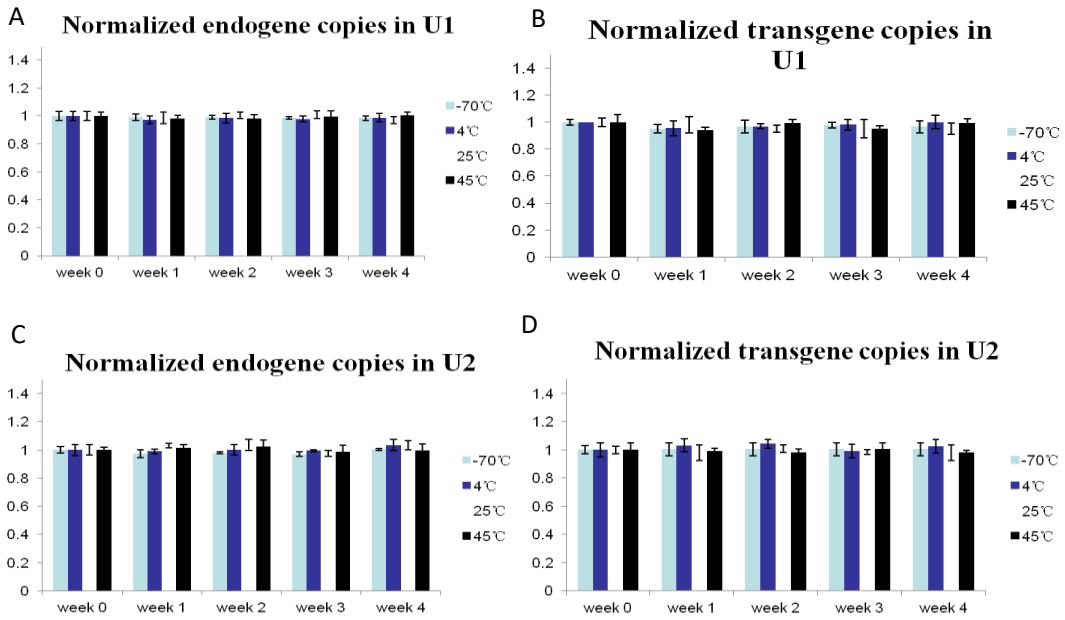


**Figure 2. Results of short term stability test.**

(A) Endogene copies in sample 1; (B) transgene copies in sample 1; (C) endogene copies in sample 2; (D) transgene copies in sample 2. T-test at the 95 % level of confidence showed no significant changes during the four weeks.

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Long-term stability was tested by storing samples at 4ºC. Trend analysis showed no significant change for both samples duringthe time the participants conducted the analysis.

## 5. Methods and instrumentation

Participants were permitted to use their own preferred methodology for the extraction of genomic DNA from samples 1 and 2. Special care was to be taken to

prepare sufficiently purified genomic DNA. Participants were advised that the unknowns could be diluted to verify the absence of PCR inhibitors in the extracted DNA. Real-time PCR was the most commonly used method to quantify DNA sequences but other technologies such as digital PCR were also used.

Participants were permitted to use any appropriate traceable calibrant which enabled them to report a copy number ratio, expressed as a percentage, between the Bt63 and *REB4* fragments measured. Participants could also measure the absolute copy number of both DNA targets by digital PCR and to provide the ratio of those two targets.

For specific detection of event Bt63 rice, a 120 bp fragment spanning the junction between the rice genomic DNA and the 3' end of the inserted sequence element as a result of *in vitro* recombination present in the genetically modified pest-resistant Bt63 rice is amplified in TaqMan® PCR [3]. To allow relative quantification of Bt63 rice (copy number ratio), a 106 bp fragment of the taxon specific rice gene (*RBE4/ SBE4*) is also amplified [4]. TaqMan® probes are used with both assays for detection of the correct amplification products.

Participants were required to determine the ratio between the copy number of the two DNA sequences in the Sample 1 and 2:

Target sequence 1 for Bt63: (120 bp)

AGAGACTGGTGATTTCAGCGGGCATGCCTGCAGGTCGACTCTAGAGGATC
CCGGACGAGTGCTGGGGCAGATAAGCAGTAGTGGTGGGGCTACGAACAT
ATTCCTTTTCCTTCTGGACGC

Target sequence 2 for *RBE4* gene: (106 bp)

GTTTTAGTTGGGTGAAAGCGGTTAGTATCTATTCCCAACCAGATATAAAAT
ATAATATATTTGTCCCCAATTGATAAAGTTTAAGGGCATTGGAAGAACTA
ACAGG

**Table 3. Analytical methods and instrumental techniques used by the CCQM-K86.b.**

| Institute | Instrument | Extraction method | Reagents | Bt63 probe | *RBE4* probe | Bt63 amplicon (bp) | *RBE4* amplicon (bp) | Calibrant used | QC used |
|---|---|---|---|---|---|---|---|---|---|
| CENAM | Fluidigm BioMark | FAST ID extraction kit | 2×Taqman Universal Mastermix | FAM/BHQ | FAM/MGB | 120 | 106 | Not needed | - |
| GLHK | ABI 7500 | E.Z.N.A. HP Plant DNA kit | AmpliTaq Gold® DNA Polymerase with Buffer I (10x) | FAM/BHQ | FAM/MGB | 120 | 106 | GBW10090 | GBW10072[a] |
| LGC | ABI 7900HT | CTAB | 2×Taqman Universal Mastermix | FAM/BHQ | FAM/MGB | 120 | 106 | GBW10090 | - |
| NIB | ABI Prism 7900 Fast | Nucleospin Food kit | 2×Taqman Universal Mastermix | FAM/BHQ | FAM/MGB | 120 | 106 | GBW10090 | - |
| NIMC | BioRad QX100 | Promega Wizard Magnetic DNA Purification System for Food | 2×ddPCR Supermix for probes | FAM/BHQ | FAM/MGB | 120 | 106 | Not needed | - |
| NIMT | ABI 7500 | (Not provided) | 2×Taqman Universal Mastermix | FAM/BHQ | FAM/MGB | 120 | 106 | GBW10090 | - |

| Institute | Instrument | Extraction method | Reagents | Bt63 probe | *RBE4* probe | Bt63 amplicon (bp) | *RBE4* amplicon (bp) | Calibrant used | QC used |
|---|---|---|---|---|---|---|---|---|---|
| NMIA | BioRad QX100 | Promega Wizard Magnetic DNA Purification System for Food | 2×ddPCR Supermix for probes | FAM/BHQ | FAM/MGB | 120 | 106 | Not needed | - |
| NMIJ | ABI ViiA7 | GM quicker 2 (NipponGene, Japan) | 2×Taqman Universal Mastermix | FAM/BHQ | FAM/MGB | 120 | 106 | GBW10090 | - |
| UME | Roche LC480 | Modified CTAB (ISO Guide 21571:2005) | LC480 Probes Master kit, 04707494001 | FAM/BHQ | FAM/MGB | 120 | 106 | GBW10090 | - |

[a] the CRM for GM Bt63 assigned on mass fraction.

Six participants carried out their measurements by quantitative PCR (qPCR); three participants reported their results using dPCR in the CCQM K86.b. 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. Only two NMIs have used the chemical DNA extraction method (CTAB), the other NMIs have all extracted the DNA using a commercial DNA extraction kits. The most used real-time PCR apparatus in the study are the Sequence Detection Systems developed by Applied Biosystems (models 7500, 7900HT and ViiA7). The ,LC 480 qPCR instrument developed by Roche was also used in the study. The absolute quantification of DNA targets was performed by four participants using either chip based Biomark system from Fluidigm or droplet based QX100 from BioRad. Five participants used the TaqMan Universal PCR Mastermix as recommended in the protocol. Platform-specific Mastermixes were used with both Roche and Bio-Rad instruments. GLHK used AmpliTaq Gold® DNA Polymerase with Buffer I rather than the TaqMan Universal PCR Mastermix.

The probes were all dual labelled using FAM and Black Hole Quenchers (BHQ1) for the event specific gene, and using FAM and Minor Groove Binder non-fluorescent quenchers (MGBNFQ) for the reference gene, as recommended in the protocol. All participants amplified the 120 bp amplicon for Bt63 and 106 bp amplicon for the *RBE4* taxon-specific gene. GBW10090 CRM was the calibrant for all participants using qPCR (Table 3)

## 6. CCQM-K86.b participants' results

The participants' measurement results are given in Tables 4 and 5. The results are shown graphically in Figures 3 and 4. The median of the study is given as an informative value in Figures 3 and 4.

For Sample 1, the two lowest KC results (LGC, NIMT) appear to differ appreciably from other participants; for Sample 2, the CENAM result appears somewhat high. The consistency plots in figure 5 and 6, which show significant pairwise differences, confirm that the two lowest results for Sample 1 differ significantly from multiple other KC participants; the remaining six results show good agreement. For Sample 2, the high CENAM result differs significantly from the UME and NMIA results.

**Table 4.CCQM-K86.b participants' measurement results for sample 1.**

| NMI/DI | Reported results $x$(cp/cp) (%) | Standard uncertainty $u$(cp/cp) (%) | Coverage factor k | Expanded uncertainty $U$(cp/cp) (%) | Relative expanded uncertainty $U/x$(%) |
|---|---|---|---|---|---|
| LGC(q) | 0.53 | 0.12 | 2.00 | 0.23 | 43 |
| NIMT(q) | 0.56 | 0.07 | 2.00 | 0.14 | 25 |
| UME(q) | 0.75 | 0.09 | 2.00 | 0.18 | 24 |
| NMIA(dd) | 0.81 | 0.05 | 2.11 | 0.11 | 14 |
| GLHK(q) | 0.88 | 0.09 | 2.00 | 0.18 | 20 |
| NIMC(dd) | 0.95 | 0.04 | 2.00 | 0.08 | 8.8 |
| NIB(q) | 0.97 | 0.08 | 2.00 | 0.16 | 16. |
| CENAM(d) | 1.01 | 0.09 | 2.00 | 0.17 | 17 |
| NMIJ(q) | 1.02 | 0.10 | 2.00 | 0.19 | 19 |

(q): determined by real time quantitative PCR; (d): determined by chip based dPCR; (dd) determined by ddPCR; (cp): copies.

**Table 5. CCQM-K86.b participants' measurement results for sample 2.**

| NMI/DI | Reported results x (cp/cp) (%) | Standard uncertainty u (cp/cp) (%) | Coverage factor k | Expanded uncertainty U(cp/cp) (%) | Relative expanded uncertainty U/x(%) |
|---|---|---|---|---|---|
| LGC(q) | 2.40 | 0.65 | 2.00 | 1.30 | 54 |
| UME(q) | 2.47 | 0.27 | 2.00 | 0.53 | 21 |
| NMIA(dd) | 2.57 | 0.14 | 2.09 | 0.28 | 11 |
| NIB(q) | 2.93 | 0.14 | 2.00 | 0.28 | 10 |
| GLHK(q) | 3.05 | 0.31 | 2.00 | 0.61 | 20 |
| NIMC(dd) | 3.05 | 0.17 | 2.00 | 0.34 | 11 |
| NMIJ(q) | 3.37 | 0.31 | 2.00 | 0.61 | 18 |
| NIMT(q) | 3.38 | 0.43 | 2.00 | 0.86 | 25 |
| CENAM(d) | 3.87 | 0.29 | 2.00 | 0.57 | 15 |

(q): determined by real time quantitative PCR; (d): determined by chip based dPCR; (dd) determined by ddPCR; (cp): copies.

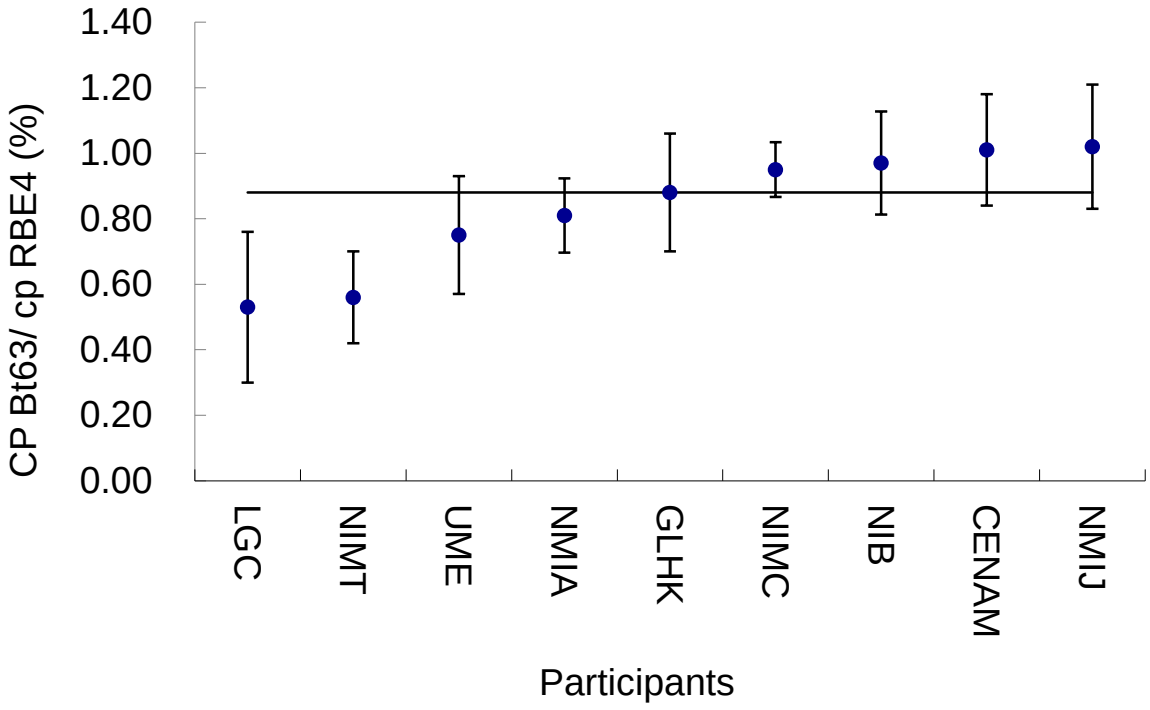


**Figure 3. Reported results with expanded uncertainties: Sample 1.**
The horizontal line shows the median (solid line).The pilot study results (red triangle) were not included in the determination of the median.

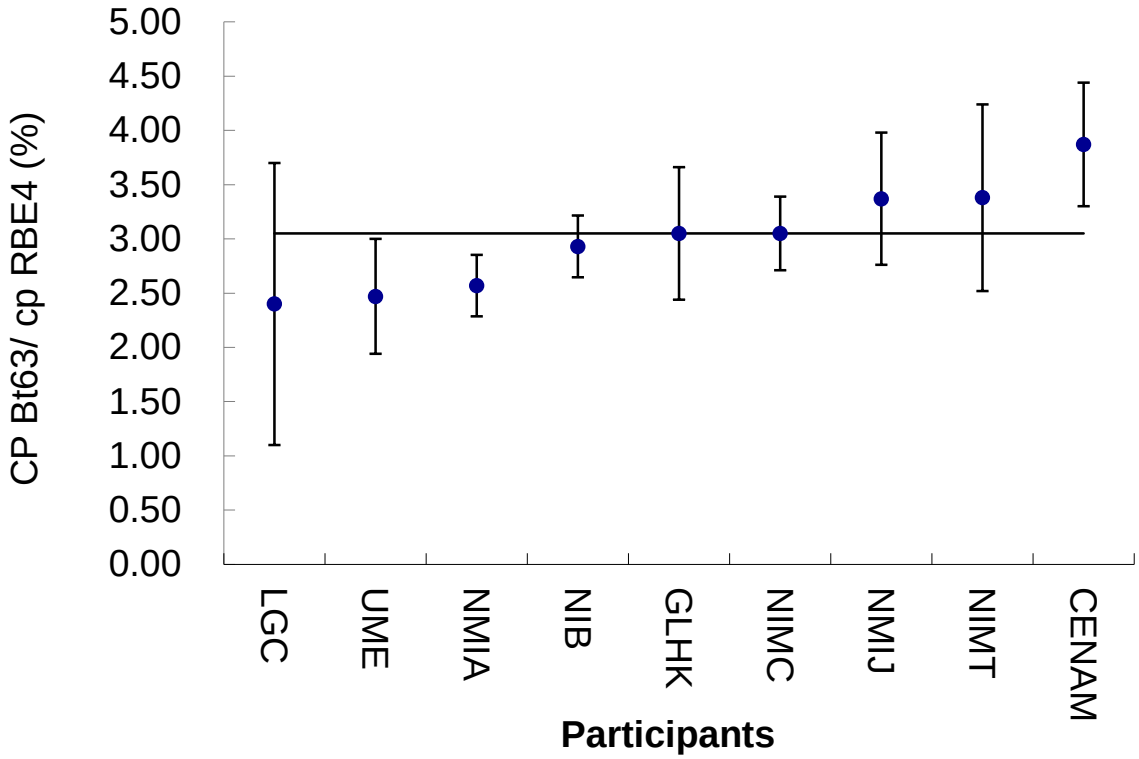


**Figure 4. Reported results with expanded uncertainties: Sample 2.**
The horizontal line represents the median (solid line).The pilot study results (red triangle) were not used for the determination of the median.

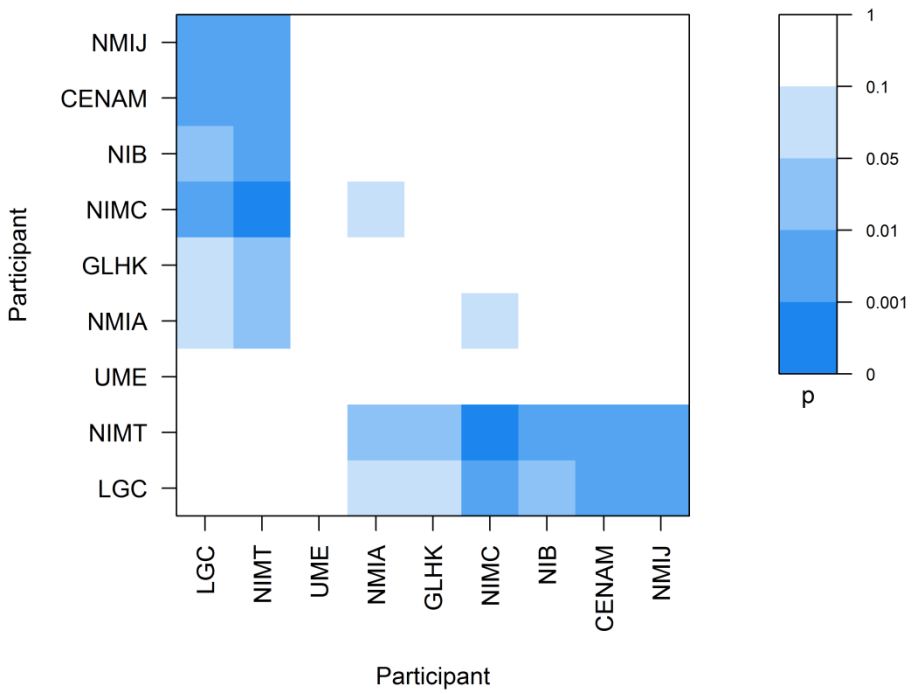


**Figure 5.Pairwise consistency plot, Sample 1.**

Colours (as key) show the p-value for a pairwise test of significance of the difference between two laboratories given their reported uncertainty.

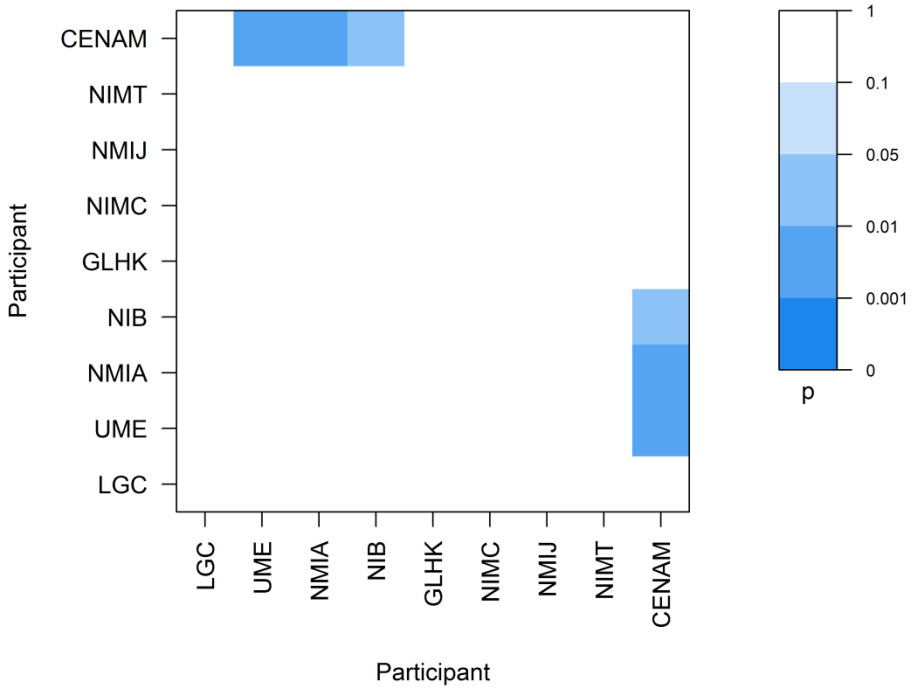


**Figure 6.Pairwise consistency plot, Sample 2.**

Colours (as key) show the p-value for a pairwise test of significance of the difference between two laboratories given their reported uncertainty.

## 7. Key comparison reference value (KCRV)

The key comparison reference value (KCRV) was estimated following the draft CCQM guidance note [5]. All CCQM-K86.b participants' result were used to calculate the KCRV. The nucleic acid working group has defined the candidate set as the reported results calibranted for copy number ratio.

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 $[x_i\text{-med}(x)]/u(x_i)$ in Figures 7and 8.The graphical inspection has been supported by outlier tests (Dixon's and Grubbs's test) performed on the reported value from participants. The result of Sample 1 from Lab4 from the pilot study was identified as an outlier at the level of 95 % confidence indicated by Dixon's and Grubbs's test.

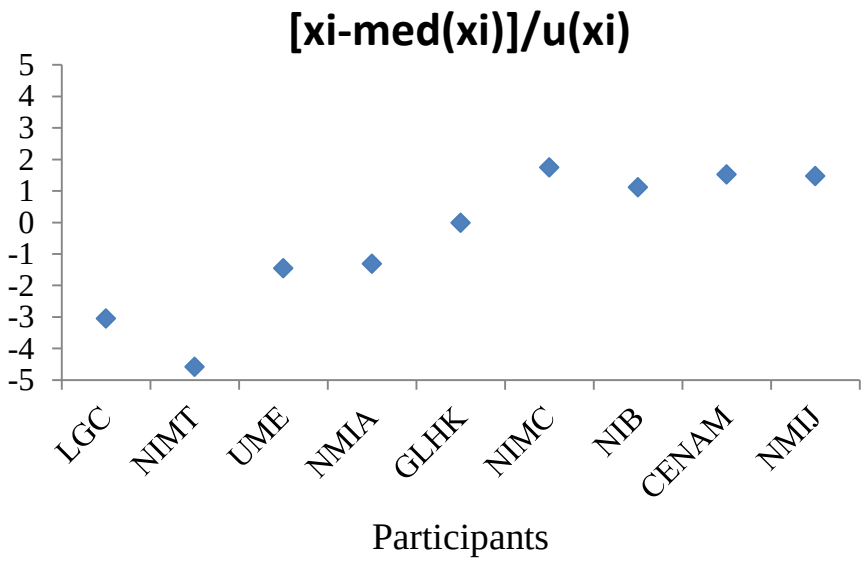


**Figure 7**: Identification of the results for Sample 1 that are far from the median relative to their uncertainties.

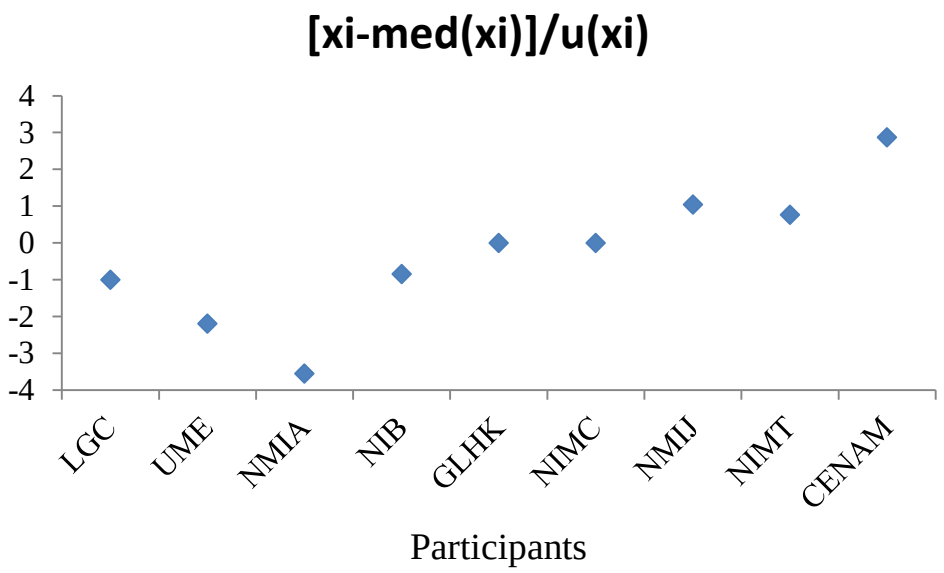


**Figure 8**: Identification of the results for Sample 2 that are far from the median relative to their uncertainties.

Extreme values were also examined by calculating a robust estimate of location $\hat{\mu}$ and dispersion$\hat{\sigma}$ of the data in figures 7 and 8, and values considered as extreme when outside $\hat{\mu} \pm 2\hat{\sigma}$ (corresponding to approximately 95 %confidence). The NIMT result for Sample 1 (as Figure 7 suggests) and the NMIA result for Sample 2 (as Figure 8 suggests) appear as outliers using this approach, indicating a possible underestimation of their uncertainties for these two results. However, they are not excluded from the KCRV determination decided by the nucleic acid working group.

A number of candidate KCRVs are compared in Table 6. The estimated KCRV values for Sample 1 vary from 0.831 to 0.880 depending on the estimator used, with a standard uncertainty between 0.026 and 0.089. The estimated KCRV values for Sample 2 vary from 2.918 to 3.050 depending on the estimator used, with a standard uncertainty between 0.093 and 0.230.

**Table 6**.Candidate KCRV estimators[Note 1, Note 2]

| Sample | Estimator | KCRV | *u* (Note 3) | $DF_{eff}$ (Note 4) | *k* | *U* | *U* (*k*=2) | Remark |
|---|---|---|---|---|---|---|---|---|
| Sample1 | Arithmetic mean | 0.831 | 0.063 | 8.569 | 2.280 | 0.143 | 0.126 | |
| | Median/MADe | 0.880 | 0.081 | 8.375 | 2.288 | 0.186 | 0.163 | |
| | DerSimonian-Laird (DSL) | 0.836 | 0.056 | 8.721 | 2.273 | 0.128 | 0.113 | Between-lab SD:0.14 |
| | Weighted mean | 0.856 | 0.026 | 12.286 | 2.173 | 0.056 | 0.052 | |
| | H15 [Note 5] | 0.831 | 0.089 | 8.282 | 2.292 | 0.203 | 0.177 | Huber(omitting $u_i$) |
| | Huber [Note 6] | 0.866 | 0.053 | 8.889 | 2.266 | 0.120 | 0.106 | Huber (including $u_i$) |
| Sample2 | Arithmetic mean | 3.010 | 0.172 | 10.225 | 2.221 | 0.382 | 0.344 | |
| | Median/MADe | 3.050 | 0.213 | 9.429 | 2.247 | 0.479 | 0.427 | |
| | DerSimonian-Laird (DSL) | 3.005 | 0.155 | 10.828 | 2.205 | 0.341 | 0.309 | Between-lab SD: 0.33 |
| | Weighted mean | 2.918 | 0.093 | 16.277 | 2.117 | 0.197 | 0.186 | |
| | H15 [Note 5] | 3.002 | 0.230 | 9.177 | 2.256 | 0.518 | 0.459 | Huber (omitting $u_i$) |
| | Huber [Note 6] | 2.904 | 0.154 | 10.657 | 2.210 | 0.339 | 0.307 | Huber (including $u_i$) |

Note 1. This list is not exhaustive.

Note 2: All values as copy number ratio expressed as a percentage

Note 3. Combined standard uncertainty $\sqrt{u_{\text{kcrv}}^2 + u_{\text{hom}}^2}$

Note 4. Effective degrees of freedom and corresponding coverage factor *k* calculated from $u_{\text{kcrv}}$ and $u_{\text{hom}}$ assuming 8 and 10 degrees of freedom respectively.

Note 5.Huber H15 estimate; a robust estimator taking no account of reported uncertainty. Typically behaves between median and mean.

Note 6. Robust estimate using reported uncertainties together with Huber weighting function. Recommended for use with generally credible uncertainties with a small number of discrepant observations.

In summary, the Graybill-deal weighted mean and H15 estimate are not recommended for the present data owing, respectively, to the evidence of excess dispersion and to the number of modest outliers affecting H15. The median and mean do not use reported uncertainties; of these two the median is recommended over the mean for its resistance to modest outlying values. If it is considered desirable to use reported uncertainty information, the Huber and DSL estimators can be considered. Of these, the Huber is to be preferred if resistance to outlying values is considered

important, while the DSL estimate is defensible otherwise.

Median was chosen for the calculation of the KCRV decided by the WG. The median is **0.88 %** and **3.05 %** for Sample 1 and Sample 2, respectively. Its uncertainty, $u(\mathrm{med}(x))$, is calculated as $[\frac{\pi}{2m}\sigma^2]^{0.5}$. $\sigma$ is the median absolute deviation mad($x$) multiplied by 1.483 where mad($x$) was 0.13 % and 0.33 % for Sample 1 and Sample 2, respectively. Here $m$ is 9 so that $u(\mathrm{med}(x))$ is 0.08 % and 0.2 % for Sample 1 and Sample 2, respectively. The relative uncertainty of the median is 9.2 % and 6.7 % for Sample 1 and Sample 2, respectively. Combining with the uncertainty of inhomogeneity, thus the relative combined uncertainty of the KCRV is 9.3 % and 7.0 % for sample 1 and sample 2, respectively. The expanded uncertainty for the KCRV, with a coverage factor $k$ of 2.288 and 2.247, is 21 % and 16 % for Sample 1 and Sample 2, respectively. The KCRV with its expanded uncertainty is shown in Figures 9 and 10 and Table 7.

Table 7. KCRV based on K86.b

| KCRV<br>$\hat{}_H$ | Bt63/rbe4<br>Copy number ratio<br>[%] | u<br>[%] | U<br>$k$*<br>[%] |
|---|---|---|---|
| Sample 1 | 0.88 | 0.08 | 0.19 |
| Sample 2 | 3.05 | 0.21 | 0.48 |

* $k$=2.288 and 2.247 for Sample 1 and Sample 2, respectively.

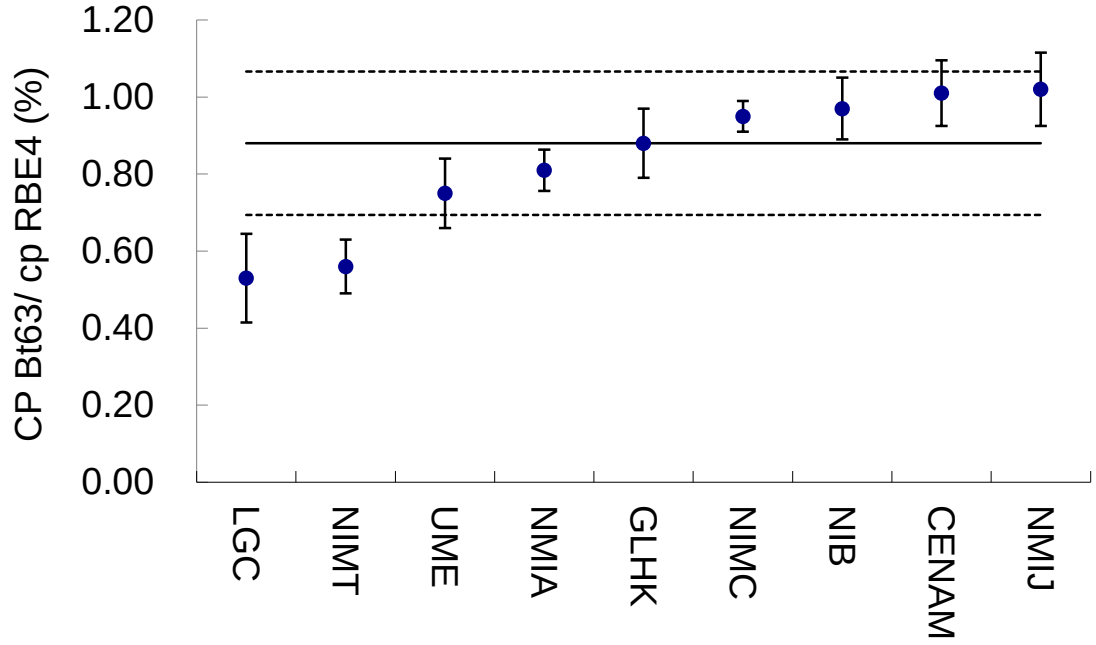


**Figure 9. Reported results and standard uncertainties: sample 1.**

Horizontal lines represent the median as the Key Comparison Reference Value (KCRV, solid line) with its expanded uncertainty (*k*=2.288) (dash line).

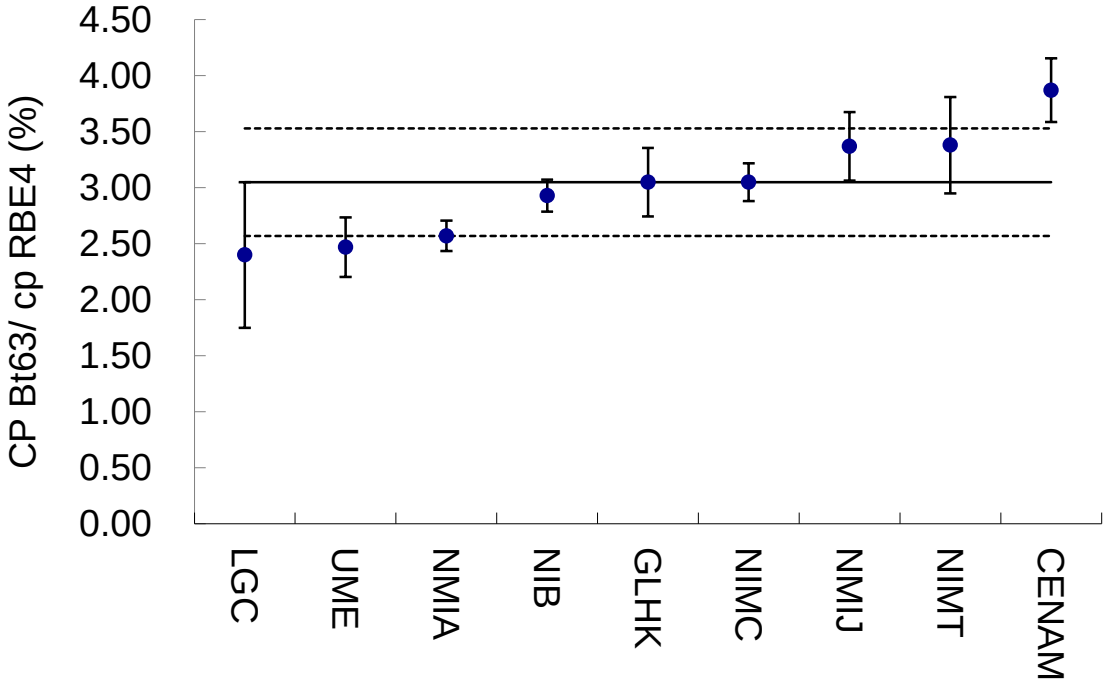


**Figure 10. Reported results and standard uncertainties: sample 2.**

Horizontal lines represent the median as the Key Comparison Reference Value (KCRV, solid line) with its expanded uncertainty (*k*=2.247) (dash line).

## 8. Degree of equivalence with respect to KCRV

The equivalence statements are calculated following the CCQM guidance note [5], which specifies that the degree of equivalence between a NMI result $x_i$ and the $x_{KCRV}$ is expressed as the difference Di calculated as:

$D_i = x_i - x_{KCRV}$

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

$$u^2(D_i) = u^2(x_{KCRV}) + u^2(x_i)$$

The degrees of equivalence with expanded uncertainties calculated as above are shown in Table 8 and illustrated graphically in Figures 11 and 12.

Table 8. Degrees of equivalence (DoE) with respect to KCRV

| Lab | Sample 1 | | Lab | Sample 2 | |
|---|---|---|---|---|---|
| | *Di* (%) | U(*Di*) (%) | | *Di* (%) | U(*Di*) (%) |
| LGC | -0.35 | 0.28 | LGC | -0.65 | 1.37 |
| NIMT | -0.32 | 0.21 | UME | -0.58 | 0.68 |
| UME | -0.13 | 0.24 | NMIA | -0.48 | 0.50 |
| NMIA | -0.07 | 0.19 | NIB | -0.12 | 0.51 |
| GLHK | 0.00 | 0.24 | GLHK | 0.00 | 0.74 |
| NIMC | 0.07 | 0.18 | NIMC | 0.00 | 0.54 |
| NIB | 0.09 | 0.23 | NMIJ | 0.32 | 0.74 |
| CENAM | 0.13 | 0.23 | NIMT | 0.33 | 0.96 |
| NMIJ | 0.14 | 0.25 | CENAM | 0.82 | 0.71 |

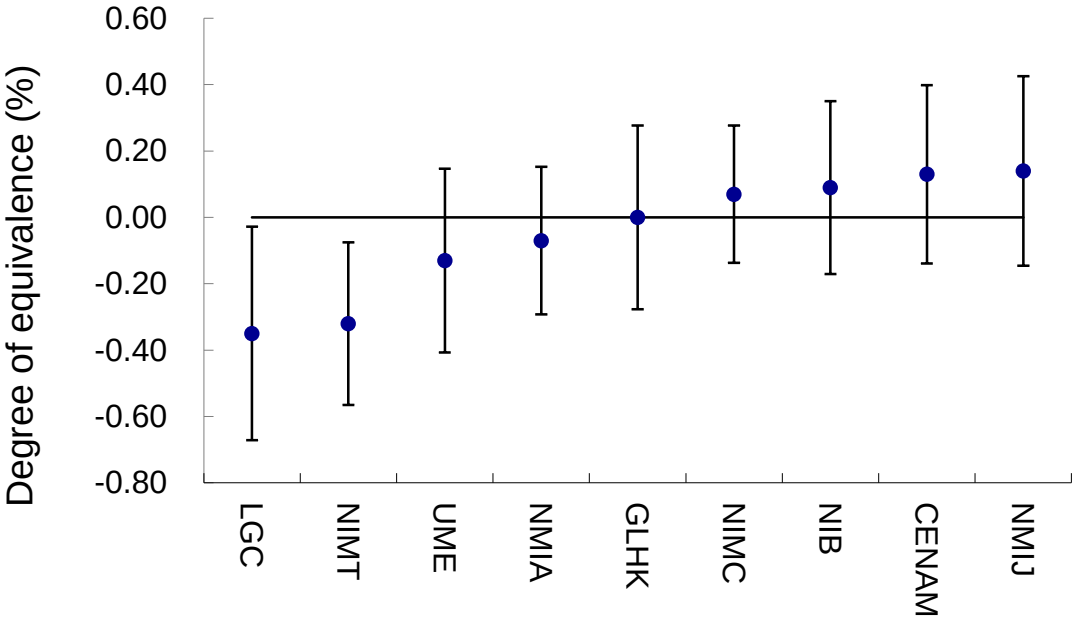


Figure 11. Degree of equivalence with expanded uncertainties with respect to KCRV for Sample 1

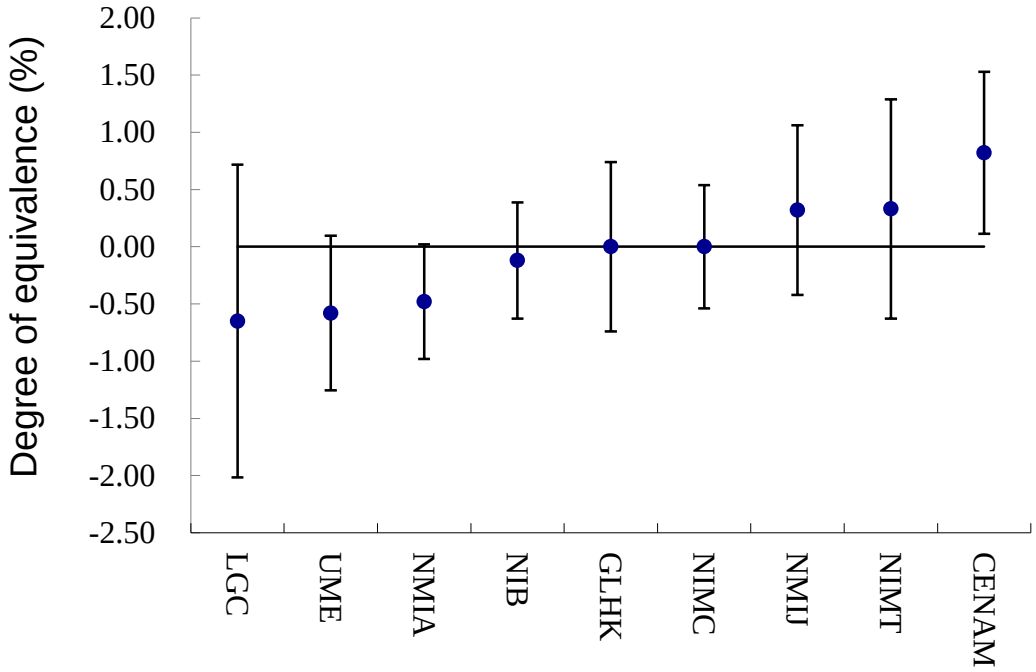


Figure 12. Degree of equivalence with expanded uncertainties with respect to KCRV for Sample 2

## 9. Discussion

Among the 9 results of the key comparison, a good agreement was observed

between the reported key comparison results. Several different extraction methods, including CTAB, modified CTAB and 5 different commercial kits, have been applied to extract the DNA from the rice tissues and led to very similar 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 target DNA fragments present in the ground rice seeds is traceable to the calibrant used, the plasmid DNA GBW10090 certified for its copy number ratio. Within the CCQM K86.b, results obtained by qPCR were in agreement with the dPCR and ddPCR results. Additionally, two ddPCR results were not significantly different from one dPCR results, suggesting no dPCR platform specificity.

All participants used the recommended primer and probe sequence amplified the 120 bp amplicon for Bt63 and 106 bp amplicon for the *RBE4* taxon-specific gene. One participant also used another primer and probe sequence targeting junction region between the cry1A(b)/cry1A(c) fusion gene and the nopaline synthase terminator (T-nos) for Bt63 [6]. However, the result of both samples from this participant was one fold higher compared with the reported corresponding KCRV. Interestingly, the whole transgene of Bt63 consists of two copies of the junction region between the cry1A(b)/cry1A(c) and T-nos and one copy of the 3' integration border region between the insert of rice event Bt63 and the rice host genome [7]. This can explain why one time higher of Bt63 content in both samples when targeting the junction region compared with border region.

CCQM-K86.b should allowed NMIs and DIs to claim measurement capabilities for the relative quantification of genomic fragments in high starch rice tissues taking into account the mentioned limitations. Some general aspects of quantification of GM material have not been addressed in CCQM-K86.b. Those concern mainly the sampling protocol, the ability to quantify DNA fragments presenting some degree of degradation and the design of primers and probes that have not been verified in this study. The CCQM-K86.b does not support the ability of either an NMI or a DI to screen for the presence of unknown GM product in a biological tissue.

## 10. Acknowledgements

Stephen L R Ellison from LGC is kindly acknowledged for his support in the data analysis. Philippe Corbisier from IRMM is acknowledged for his support in the drafting the report. David Lee Duewer from NIST is also acknowledged for providing the PDF_Maker Total exploratory consensus assessment tool.

## 11. References

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[3] Wu G, Wu Y, Nie S, Zhang L, Xiao L, Cao Y, Lu C. Real-time PCR method for detection of the transgenic rice event TT51-1. Food Chemistry, 2010, 119 (1), 417-422.

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[5] CCQM Guidance note: Estimation of a consensus KCRV and associated Degree of Equivalence.Version 10, Date: 2013-04-12

[6] CRL-EM-02/06, Report on verification of the performance of a method for the detection of “Bt63” rice using real time PCR, 2008.

[7] Patent 200810048245.6, CN 101302520A, Sequence of integration site of the exogenous vector and application of the genetically modified TT51-1 event specific rice.

## Appendix A: Study protocol

# CCQM key comparison (CCQM-K86.b) “Relative quantification of Bt63 in GM rice matrix sample”

### Draft Technical Protocol

## Background

Rice is an important grain in rice-growing nations and is consumed in large quantities. In order to improve the yield of rice and to decrease the use of pesticides, some GM varieties of rice are developing all over the world. Bt63 is genetically modified pest-resistant rice.

Bt63 rice has been produced by Huazhong Agriculture University in China. In August 2009, Bt63 rice obtained the safety permission certificate issued by the Ministry of Agriculture, China. In order to protect rice businesses in countries in which Bt63 rice is not authorized, Bt63 rice should be screened in all exported rice. Additionally, the measurement accuracy of Bt63 rice test is very important. Therefore, reference materials for Bt63 rice are needed to ensure that the screening and quantification tests are accurate, reliable, and comparable.

This study (CCQM K86-b), discussed and agreed at the 26th CCQM BAWG meeting in Tsukuba, Japan in 2014, aims to extend CMCs supported by CCQM K86, to include a higher starch matrix. In this proposed study, Bt63 rice and wild type (wt) rice will be mixed in different ratios and the copy number ratio of event specific gene and endogenous gene in the genomic DNA extracted from the Bt63 rice matrix need to be determined.

## Description of the Measurands

Two unknown samples are rice powders each containing different copy number ratio of Bt63 gene to endogenous gene.
The measurand is a relative amount of DNA that has been quantified by determining the copy number ratio between the Bt63 gene and the endogenous

gene.

## Preparation of Study Materials

Two rice powders each containing a defined mass fraction of genetically modified Bt63 rice were prepared as the study materials for this pilot study. The samples were produced under the responsibility of the NIM and were prepared by mixing of dried non-GM rice powder and dried Bt63 GM rice powder at different ratios.

For specific detection of event Bt63 rice, 120 bp fragment of the single copy DNA integration-border region of the genomic sequence and the 3' end of the inserted sequence element as a result of *in vitro* recombination present in the genetically modified pest-resistant Bt63 rice is amplified in TaqMan® PCR. For relative quantification of Bt63 rice (copy number ratio), a 106 bp fragment of the taxon specific rice gene (*RBE4/ SBE4*) using a gene specific combination of primers and probe is amplified.

A calibrant (RM-pBt63) for the real-time PCR quantification (qPCR) will be provided by NIM. The pBt63 was processed according to the ISO Guide 34. The RM is certified for the number of DNA fragments per plasmid of a Bt63 transgenic sequence and of the endogenous gene (*RBE4*) as 1:1. CRM (GBW10092) is intended to be used for the calibration of Bt63 rice with qPCR method.
The ratio between the copy number of those two DNA sequences in the sample 1 and
2 must be determined:
Target sequence 1 for Bt63: (120 bp)
AGAGACTGGTGATTTCAGCGGGCATGCCTGCAGGTCGACTCTAGAGGATCCCGGA
CGAGTGCTGGGGCAGATAAGCAGTAGTGGTGGGGCTACGAACATATTCCTTTTCCT
TCTGGACGC
Target sequence 2 for RBE-4 gene: (106 bp)
GTTTTAGTTGGGTGAAAGCGGTTAGTATCTATTCCCAACCAGATATAAAATATAATAT
ATTTGTCCCCAATTGATAAAGTTTAAGGGCATTGGAAGAACTAACAGG
Participants are also welcome to use any other types of calibrant which they think enable them to report a copy number ratio, expressed in percent, between the Bt63 and *REB4* fragments measured. Participants can also measure the absolute number of both DNA targets by digital PCR and to provide the ratio of those two numbers.

## Methodology

Participants can use their own 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. Real-time PCR is the most commonly used method to quantify DNA sequences but other technologies such as digital PCR, can also be used.

## Homogeneity testing

Homogeneity analysis of the DNA sequences in sample 1 and 2 has been performed by NIM and the uncertainty related to the homogeneity will be provided. The sample intake used for determining the homogeneity was 100 mg.

## Stability testing

The study materials have sufficient short (-70ºC, 4ºC, 25 ºC and 45 ºC) and long-term (-70 ºC and 4ºC) stability which was tested by NIM.

## Instructions for use

Participants will receive 2 samples in glass bottles each containing at least 1 g of sample, and 1 tube with 100 µl of CRM-GBW10092 solution. Samples and CRM-GBW10092 will be sent in dry ice. Samples 1 and 2 should be stored at 4 ºC and CRM-GBW10092 should be stored at -20 ºC or below upon arrival.

## Result reporting

A reporting template and detailed questionnaire will be provided to the participants. The participants shall submit the questionnaire by e-mail to donglh@nim.ac.cn and wj@nim.ac.cn. Additionally, 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 donglh@nim.ac.cn and wj@nim.ac.cn. Raw data of the preparation of the calibration curves and unknowns should be provided. The results indicating the relative percentage of both sequences 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.

Results returned should include,

1. The ratio of target Bt63 to target RBE-4 expressed in percentage for samples 1 and 2 as well as the expanded uncertainty.
2. An outline of the methodology, a measurement equation and a breakdown of the uncertainty estimation should be submitted.

## Proposed Timetable

1. Distribution of draft testing protocol and call for participation: 30th **June, 2015**
2. Sign up for participation: **15th July, 2015**
3. Shipping of test materials and calibrator: **10th Sep, 2015**
4. Return of the measurement results: **1st Dec, 2015**
5. Draft Report: **April, 2016**
6. Circulation of final draft report: **Oct, 201**6