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Comparative Testing Report on the Detection and Quantification of Maize

Comparative testing round: ILC-CRL-GMFF-CT-01/10

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The mission of the JRC-IHCP is to protect the interests and health of the consumer in the framework of EU legislation on chemicals, food, and consumer products by providing scientific and technical support including risk-benefit assessment and analysis of traceability.

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Comparative Testing Report on the Detection and Quantification of Maize Event NK603

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Confidentiality statement: The laboratory codes assigned to each participant in this comparative testing round are confidential. However, the EURL-GMFF will disclose details of the National Reference Laboratories that have been appointed under Regulation (EC) No 882/2004 and Regulation (EC) No 1981/2006 to DG SANCO for the purpose of an assessment of their performance.

Executive Summary

The Joint Research Centre as European Union Reference Laboratory for Genetically Modified Food and Feed, established by Regulation (EC) No 1829/2003[(1)], organised a comparative testing round for National Reference Laboratories nominated under Regulation (EC) No 882/2004[(2)] and Regulation (EC) No 1981/2006[(3)] and for laboratories from third countries that volunteered to participate.

In accordance with Article 32 of Regulation (EC) No 882/2004 of the European Parliament and of the Council of 29 April 2004 on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules, the European Union Reference Laboratory for Genetically Modified Food and Feed shall organise comparative testing and shall ensure an appropriate follow-up of such testing.

The design and execution of the comparative testing round was in accordance with the ISO 17043 standard[(4)]. The European Union Reference Laboratory for Genetically Modified Food and Feed is in the process to become ISO 17043 accredited.

The test items used in the comparative testing round ILC-CRL-GMFF-CT-01/10 were produced by the Reference Materials Unit of the Institute for Reference Materials and Measurements (Geel, Belgium). Participants had to determine the genetically modified (GM) content in two test items denoted maize powder level 1 and level 2, containing different GM percentages of maize event NK603 flour. Maize powder level 1 was a 0.1 % GM Certified Reference Material that was re-labelled. Maize powder level 2 was a 1.7 % GM NK603 flour produced under validated processing conditions but never released as a Certified Reference Material. In January 2010, a total of 110 laboratories were invited to participate in ILC-CRL-GMFF-CT-01/10. Five National Reference Laboratories declined participation. Test items were shipped to the participants beginning of March 2010 in dark brown glass bottles containing approximately 1 g of flour. Ninety-three results were returned from 84 laboratories from 36 countries, of which 27 were National Reference Laboratories nominated under Regulation (EC) No 1981/2006, five were National Reference Laboratories nominated under Regulation (EC) No 882/2004, 34 were National Reference Laboratories nominated under both Regulations, seven were members of the European Network of GMO Laboratories only and 11 were laboratories from third countries. Five laboratories including two National Reference Laboratories, two European Network of GMO Laboratories only members and one laboratory from a third country did not submit any results. The Food Safety and Quality Unit of IRMM managed the on-line registration and submission of results.

Participants could report the results of the exercise either in mass/mass % or in copy/copy %. For the data expressed in mass/mass % the assigned values (μ) and associated uncertainties were provided by the Reference Materials Unit of IRMM. For maize powder level 1 the assigned value and associated uncertainty of the certificate were taken. For maize powder level 2, data

from the homogeneity study conducted at the European Union Reference Laboratory for Genetically Modified Food and Feed's premises were included in the uncertainty budget. In addition, the European Union Reference Laboratory for Genetically Modified Food and Feed calculated the robust means ($\hat{\mu}$) of the maize powder level 1 and level 2 test items in mass/mass % and in copy/copy %. All data were log-transformed and robust statistics was applied to obtain a robust mean [5, 6, 7].

The target standard deviation for comparative testing $\hat{\sigma}$ for maize event NK603 was fixed to 0.25 ($\log_{10}$ value) by the Advisory Board for Comparative testing on the basis of the state-of-the-art in this field of analysis. This target standard deviation was used to derive z-scores for the participants' results. An overview of the assigned values, robust means and number of z-scores in the range of -2 to +2 is given in Table 1.

Table 1. Reference values expressed in mass/mass % (m/m %) and copy/copy % (cp/cp %), and share of z-scores in the range of -2 to +2

	Result	No. of scores, working range ($\lvert z \rvert \leq 2$)	No. of scores outside working range, ($\lvert z \rvert > 2$)	Total no. of scores
GM level 1	0.10[1]	48	2	50
GM level 2	1.69[1]	58	0	58
GM level 1	0.12[2]	48	2	50
GM level 2	1.69[2]	58	0	58
GM level 1	0.10[3]	25	4	29
GM level 2	1.47[3]	32	2	34

[1] Assigned value (m/m %)
[2] Robust mean (m/m %) calculated as $10^{\text{robust mean (log (value))}}$
[3] Robust mean (cp/cp %) calculated as $10^{\text{robust mean (log (value))}}$

The outcome of this first comparative testing round was in general positive with a share of 86-96 % and 94-100 % of participants exhibiting a z-score in the range of -2 to +2 for maize powder level 1 and level 2, respectively.

Drafted by:

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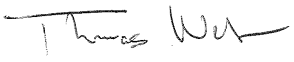
T. Weber (Sampling and statistics officer) 

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Content

1. Introduction

The Joint Research Centre (JRC) as European Union Reference Laboratory for Genetically Modified Food and Feed (EURL-GMFF) was established by Regulation (EC) No 1829/2003[1] of 22 September 2003 on genetically modified food and feed. The EURL-GMFF has two mandates determined by Regulation (EC) No 1981/2006[3] of 22 December 2006 on detailed rules for the implementation of Article 32 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council as regards the Community reference laboratory for genetically modified organisms (GMOs) and by Regulation (EC) No 882/2004[2] of the European Parliament and of the Council of 29 April 2004 on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules.

In accordance with Article 32 of Regulation (EC) No 882/2004 the EURL-GMFF shall organise comparative testing for National Reference Laboratories (NRLs) and shall ensure an appropriate follow-up of such testing. The aim of this activity is 'to contribute to a high quality and uniformity of analytical results'[2]. Moreover, Article 12 of Regulation (EC) No 882/2004 states that the nominated NRLs should be accredited in accordance with ISO/IEC 17025 on 'General requirements for the competence of testing and calibration laboratories'. One of the requirements of ISO/IEC 17025 accredited laboratories is to prove their competence by taking part in a proficiency testing scheme.

Regulation (EC) No 1829/2003 establishes a threshold for labelling of food and feed products consisting of or containing more than 0.9 % genetically modified organisms (GMOs) provided the GMO has undergone the authorisation procedure in accordance with European Union legislation. This threshold of 0.9 % for labelling is used by the Member States of the European Union involved in the official control of food and feed. Hence, a proper determination of the GM content in sampled products is of paramount importance.

The EURL-GMFF organised the first comparative testing round in 2010 in collaboration with the Reference Materials (RM) Unit and the Food Safety and Quality (FSQ) Unit of IRMM. The comparative testing round was announced at the NRL workshop on 28 and 29 May 2009 and at the ENGL meeting on 15 December 2009. In January 2010, a total of 110 laboratories were invited to participate in ILC-CRL-GMFF-CT-01/10. Five NRLs declined participation. Test items were shipped between 8 and 10 March 2010. The deadline for submission of results was 23 April 2010. The FSQ Unit of IRMM managed the on-line registration and submission of results employing a database of the International Measurement Evaluation Programme (IMEP). Ninety-three results were returned from 84 laboratories from 36 countries, of which 27 were NRLs nominated under Regulation (EC) No 1981/2006, five were NRLs nominated under Regulation (EC) No 882/2004, 34 were NRLs nominated under both Regulations, seven were members of the European Network of GMO Laboratories only (ENGL) and 11 were laboratories from third countries. Five laboratories including two NRLs, two ENGL only members and one laboratory from a third country did not submit any results.

2. Description of comparative test items

2.1 Preparation

Test items were prepared by the RM Unit of IRMM. The RM Unit produced test items for comparative testing according to ISO Guide 34[8] regarding the 'General requirements for the competence of reference material producers'.

Maize powders were prepared by a two-step grinding process using a high impact mill[9]. Test items were obtained by turbula-mixing and dry-mixing of non-modified maize powder and NK603 maize powder. A 10 % GM mix was produced first using 100 % GM and non-GM base material. All lower concentrations were achieved by further dilution with non-GM maize powder. Powders were weighed using a calibrated balance.

Approximately 1 g of the dry-mixed test items were bottled in 10-mL brown glass vials using an automatic sampling device, under argon and re-labelled as maize powder level 1 and level 2. Maize powder level 1 was a 0.1 % GM Certified Reference Material (CRM) that was re-labelled to avoid identification by the participants as an existing CRM. Maize powder level 2 was a 1.7 % GM NK603 flour produced under validated processing conditions but never released as a CRM. Test items were stored at +4 °C in the dark.

2.2 Homogeneity and stability assessment

The assessment of the homogeneity was performed after the test items had been packed in their final form and before distribution to participants[10]. As one of the test items is a CRM, its homogeneity was assessed upon its production.

The within bottle standard deviation s_a and the among-bottle standard deviation s_s of samples is determined by employing a single factor ANOVA[11]. Samples are considered to be adequately homogeneous if:

$$s_s \leq 0.3\hat{\sigma} \qquad (1)$$

If this criterion is met, the among-bottle standard deviation contributes no more than about 10 % to the standard deviation for comparative testing.
If this criterion is not met, the among-bottle standard deviation is included in the standard deviation for comparative testing:

$$\hat{\sigma}_1 = \sqrt{\hat{\sigma}^2 + s_S^2} \qquad (2)$$

The repeatability of the test method is the square root of mean sum of squares within bottles MS_{within}, the among-bottle standard deviation s_s is given by $\sqrt{MS_{among} - MS_{within}/n}$ where MS_{among} is the mean sum of squares among bottles and n is the number of replicates. If $MS_{within} > MS_{among}$, then:

$$s_s = u^*_{bb} = \frac{repeatability}{\sqrt{n}} \sqrt[4]{\frac{2}{N(n-1)}} \quad (3)$$

where u^*_{bb} is the maximum uncertainty contribution that can be obtained by the hidden heterogeneity of the material.
Ten brown glass vials ($N = 10$) were randomly selected and analysed in five-fold replicates ($n = 5$) for maize powder level 2. The criterion described in formula (1) was fulfilled thus indicating that the maize powder level 2 test item was homogeneous.
The data from the homogeneity study conducted at the EURL-GMFF were used for the estimation of the uncertainty contributions related to the heterogeneity and to the stability of the maize powder level 2 test item.

3. Participants' results

The assignment of a laboratory number to each participant and the submission of results were managed by the FSQ Unit of IRMM. Results had to be reported on-line using a form for which each participant received an individual access code. A questionnaire was attached to the on-line reporting form to provide details of the analytical methods used.

Participants could report the results of the exercise either in mass/mass % (m/m %) or in copy/copy % (cp/cp %). The expression of measurement results in cp/cp % follows the Recommendation (EC) No 2004/787[12]. It is recommended that the results of quantitative analyses are expressed as GM DNA copy numbers in relation to target taxon-specific copy numbers calculated in terms of haploid genomes.

Participants were instructed to apply the formulas described below when reporting their results.

$$\text{m/m \%} = \frac{\text{mass GM [g]}}{\text{Total mass [g]}} \times 100\ \% \quad (4)$$

$$\text{cp/cp \%} = \frac{\text{GM DNA copy numbers [cp]}}{\text{Target taxon-specific DNA copy numbers [cp]}} \times 100\ \% \quad (5)$$

A total of 84 laboratories from 36 countries reported results (Figure 1). Fifty-nine laboratories reported the GM content in m/m % whereas 34 laboratories expressed their results in cp/cp %

(Figure 2). Eight laboratories reported the results in both measurement units. Five laboratories including two NRLs, two ENGL only members and one laboratory from a third country did not submit any results. Both NRLs gave a reason for not reporting the results. One NRL stated that it had no quantitative method available for maize event NK603. The other reported a lack of appropriate reagents for quantitative analyses. The laboratory from a third country had problems importing the test items and received the test items only after the expiry of the deadline for reporting. Both ENGL only members did not give any justification for not reporting results.

Figure 1: Distribution of participants from different countries

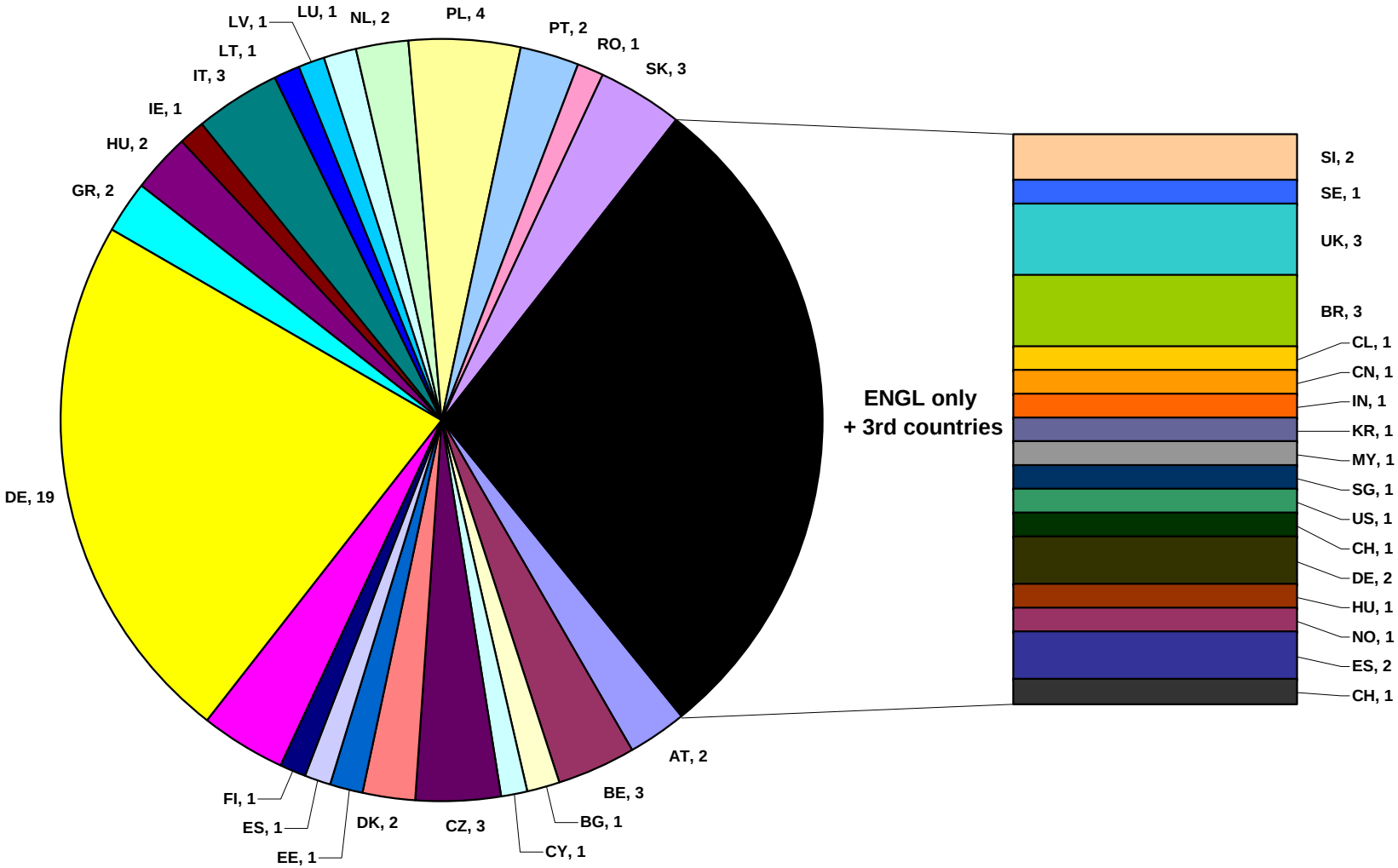
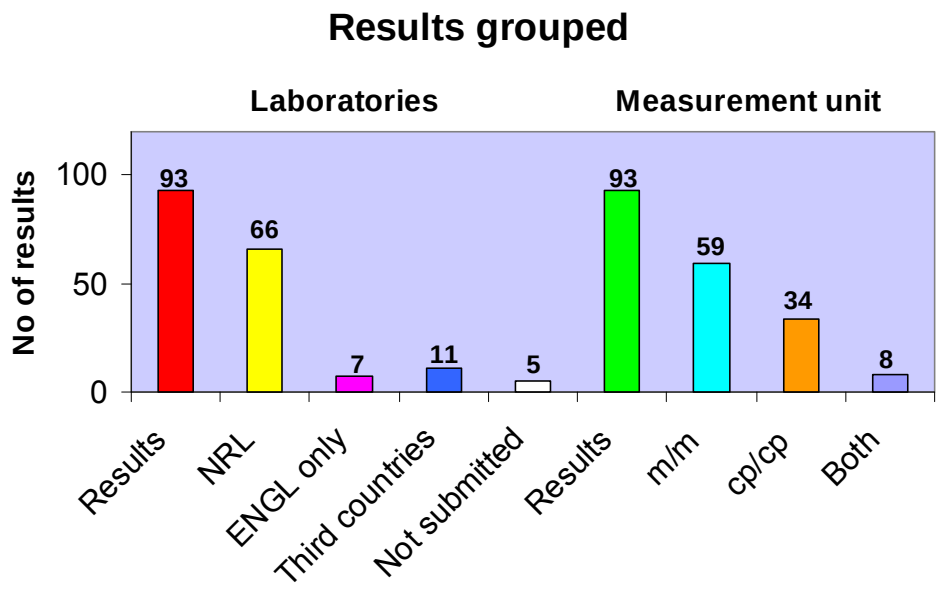


Figure 2. Overview of participants' results grouped per type of laboratory and measurement unit, respectively.



For the data expressed in m/m % the assigned values (μ) and associated uncertainties were provided by the RM Unit of IRMM. For maize powder level 1 the assigned value and associated uncertainty of the certificate were taken. For maize powder level 2, data from the homogeneity study conducted by the EURL-GMFF were included in the uncertainty budget. In addition, the EURL-GMFF calculated the robust means ($\hat{\mu}$) of the maize powder level 1 and level 2 test items in m/m % and in cp/cp %. All data were log-transformed and robust statistics were applied to obtain a robust mean[5, 6, 7].

An overview of the results reported in m/m % and cp/cp % is given in Tables 4 to 7. An overview of the analytical methods used by each participant is summarised in the section on 'Questionnaire data'.

4. Assigned value and measurement uncertainty

4.1 Reference value determined by the test item producer

The assigned value (μ) determined by the RM Unit of IRMM is based on the mass fraction of non-GM and GM powder mixed and corrected for the water content[9].

The information related to the CRL-GMFF-CT-01/10 maize powder level 1 and level 2 test items can be found in the table below.

Table 2. Assigned value μ and expanded uncertainty of maize powder level 1 and level 2

NK603 maize content [g/kg]		Standard uncertainty						Combined uncertainty	Expanded uncertainty
		(u_1)[1]	(u_2)[2]	(u_3)[3]	(u_4)[4]	(u_5)[5]	(u_6)[6]	(u_c)	$(U = 2 * u_c)$
Maize powder level 1[7]	1.0	0.0029	0.0014	0.0814	0.1732	0.004		0.1914	0.3829
Maize powder level 2[8]	16.9	0.0364	0.0006	0.5084	0.0866	0.293	0.1864	0.623	1.246

[1] Mass determination uncertainty introduced, mainly based on the uncertainty of the balance
[2] Water content average standard deviation 0.08 %, three and two dilution steps taken into consideration for maize powder level 1 and level 2, respectively.
[3] Heterogeneity at 100 mg level with an average particle size of 111 µm and a density of 0.94 g/mL
[4] Purity of non-GM base material
[5] Purity of GM base material
[6] Stability estimated to be 1.1 % relative u_{lts} for 12 months (based on comparable maize materials)
[7] Maize powder level 1 refers to a certified CRM content [g/kg](9)
[8] Maize powder level 2 refers to a comparative testing assigned content [g/kg]

The rounded certified values expressed in m/m % are: 0.1 +/- 0.04 % and 1.69 +/- 0.13 % for maize powder level 1 and maize powder level 2, respectively.

The expanded uncertainty of the certified value (U_{CRM}) comprises standard uncertainty contributions from the characterisation, the heterogeneity, and the stability(13).

$$U_{CRM} = k\sqrt{u_{char}^2 + u_{bb}^2 + u_{lts}^2} \quad (6)$$

The combined standard uncertainty comprises contributions from the characterisation of the material (u_{char}), the among-vial heterogeneity (u_{bb}) at the recommended sample intake of 100 mg and the long-term stability of the material (u_{lts}). The uncertainty contribution from the characterisation of the material includes uncertainties related to the weighing procedure, the determination of the water content in the powders, and the purity of the non-GM and GM base materials (Table 2). In the case of the maize powder level 1 test item, the uncertainty contribution from the long-term stability of the material was not taken into consideration, as it is negligible compared to the uncertainty contribution of the non-GM base material. A coverage factor of 2 was used to calculate the expanded uncertainty corresponding to a 95 % level of confidence(14).
The certified and assigned values of maize powder level 1 and level 2 are traceable to the International System of Units (SI). The traceability chain is based on the use of calibrated balances and a thorough control of the weighing procedure.

4.2 Consensus value from participants

The consensus value ($\hat{\mu}$) from participants in the comparative testing round was calculated by means of robust statistics[15]. This approach minimises the influence of outlying values. All results were log-transformed prior to the calculation of the robust mean to establish a near-normal distribution allowing the interpretation of results on the basis of the normal distribution[6]. Two robust means ($\hat{\mu}$) were calculated on the basis of the results reported in m/m % and cp/cp %, respectively.

The uncertainty of the characterisation is assessed during the comparative testing round comparison by estimating the relative standard deviation (RSD) of the robust mean. The standard uncertainty (u_{char}) of the characterisation is calculated using the formula:

$$u_{char} = \frac{RSD}{\sqrt{N}} \qquad (7)$$

where RSD = relative standard deviation of the robust mean and *N* = number of data points.

The value of the robust mean is traceable to the measurement unit of the reference material that was used for the preparation of the standard curves.

The assigned values (μ) by the test item producer and the robust means ($\hat{\mu}$) determined by the EURL-GMFF are depicted in Table 3.

Table 3. Overview of assigned values and robust means and expanded uncertainties for maize powder level 1 and level 2

Maize powder level 1	**m/m [%]**	***U*[1]**	**cp/cp [%]**	***U*[1]**
Assigned value	0.10	0.04	-	-
Robust mean	0.12[2]	0.09	0.10[3]	0.07
Maize powder level 2				
Assigned value	1.69	0.13	-	-
Robust mean	1.69[4]	1.32	1.47[5]	1.10

[1] *U* refers to an expanded uncertainty with a coverage factor *k* equal to 2 corresponding to a level of confidence of 95 %[(14)]

[2] Robust mean calculated on the basis of *N* = 50. Eight laboratories reporting the GM content as '< value x' and one laboratory that did not perform quantitative analyses were excluded from the calculation of the robust mean.

[3] Robust mean calculated on the basis of *N* = 29. Three laboratories reporting the GM content as '< value x' and two laboratories reporting a GM content equal to zero were excluded from the calculation of the robust mean.

[4] Robust mean calculated on the basis of *N* = 58. One laboratory that did not perform quantitative analyses was excluded from the calculation of the robust mean.

[5] Robust mean calculated on the basis of *N* = 34.

5. Statistical data and summaries

The aim of a performance statistic is to provide participants with a meaningful result that can be easily interpreted. The procedure followed for the evaluation of participants' performance was agreed by the Members of the Advisory Board and relies on the calculation of z-scores on the basis of the assigned value by the test item provider and the robust mean of the participants' results [(10)].

Laboratories are compared on the basis of z-scores calculated from log-transformed data[(6)]. Two types of z-scores are used, one based on the assigned value (μ) of the test item and the other based on the robust mean ($\hat{\mu}$) of the submitted results. Results reported in m/m % are analysed using both types of *z*-scores, for results reported in cp/cp %, only the robust mean is used to calculate a z-score.

The value of $\hat{\sigma}$, the target standard deviation for comparative testing, determines the performance limits in a comparative test and is set at a value that reflects best practice for the analysis in question. For this round the Members of the Advisory Board chose a value of 0.25[(16)]. The z-score (z_i) for participant i reporting measurement result x_i is thus calculated as

$$z_i = \left(\log_{10} x_i - \mu \right) \Big/ \hat{\sigma} \text{ or as } z_i = \left(\log_{10} x_i - \hat{\mu} \right) \Big/ \hat{\sigma} \qquad (8)$$

Table 4. Reported results (m/m %) and z-scores for event NK603 maize powder level 1

Maize event NK603

Laboratory number	Assigned value = 0.10 m/m % Robust mean = 0.12 m/m %				
	Value	LOD m/m	LOQ m/m	z-score[1]	z-score[2]
L01	0.11	-	-	0.17	-0.13
L02	0.23	0.09	0.28	1.45	1.15
L04	0.11	0.25	0.50	0.17	-0.13
L06	0.10	-	-	0.00	-0.30
L07	<0.1	0.04	0.10	no z-score attributed	no z-score attributed
L08	0.20	-	-	1.20	0.90
L09	<0.1	0.10	0.10	no z-score attributed	no z-score attributed
L10	<0.1	0.10	0.10	no z-score attributed	no z-score attributed
L11	<0.1	0.10	0.10	no z-score attributed	no z-score attributed
L12	0.16	0.03	0.10	0.82	0.52
L14	0.15	0.50	0.50	0.70	0.40
L15	0.11	0.02	0.10	0.17	-0.13
L18	0.14	-	-	0.58	0.28
L19	0.09	0.01	0.04	-0.18	-0.48
L20	<0.1	-	-	no z-score attributed	no z-score attributed
L22	0.10	0.02	0.07	0.00	-0.30
L23	<0.27	0.08	0.18	no z-score attributed	no z-score attributed
L24	0.20	0.00	0.00	1.20	0.90
L25	0.20	0.02	0.10	1.20	0.90
L26	0.06	0.01	-	-0.89	-1.19
L27	0.05	-	-	-1.20	-1.50
L28	0.11	0.03	0.10	0.17	-0.13
L29	0.11	-	-	0.17	-0.13
L30	0.11	0.01	0.01	0.17	-0.13
L31	0.09	-	-	-0.18	-0.48
L32	0.43	-	-	2.53	2.23
L34	0.14	-	-	0.58	0.28
L38	0.10	-	-	0.00	-0.30
L39	0.10	-	-	0.00	-0.30
L40	0.09	-	-	-0.18	-0.48
L41	0.08	0.10	-	-0.39	-0.69
L43	0.17	-	-	0.92	0.62
L47	0.14	0.01	0.10	0.58	0.28
L50	0.12	0.05	0.10	0.32	0.02
L53	0.12	0.05	0.10	0.32	0.02
L58	0.10	0.01	0.10	0.00	-0.30
L59	0.07	0.14	0.45	-0.62	-0.92
L60	0.08	0.02	0.05	-0.30	-0.60
L61	0.14	0.05	0.10	0.58	0.28
L62	0.11	-	-	0.13	-0.17
L63	0.12	0.05	0.10	0.32	0.02
L64	<0.1	0.10	0.10	no z-score attributed	no z-score attributed
L65	0.18	0.05	0.10	1.02	0.72
L66	0.29	0.10	0.05	1.85	1.55
L68	0.11	-	-	0.10	-0.20
L69	0.16	0.05	0.10	0.82	0.52
L70	0.12	0.10	0.10	0.32	0.02
L72	0.10	0.01	0.07	0.00	-0.30
L74	<0.16	0.16	0.33	no z-score attributed	no z-score attributed
L76	<0.1	0.10	0.10	no z-score attributed	no z-score attributed
L78	0.09	0.02	0.06	-0.18	-0.48
L80	0.46	0.10	0.10	2.65	2.35
L82	0.13	0.10	0.10	0.46	0.16
L83	0.10	0.02	0.09	0.00	-0.30
L84	0.12	0.05	0.10	0.32	0.02
L85	0.07	-	-	-0.62	-0.92
L86	0.11	-	-	0.23	-0.07
L87	0.13	-	-	0.46	0.16
L89	0.10	-	-	0.00	-0.30

[1] z-score calculated on the basis of the assigned value

[2] z-score calculated on the basis of the robust mean

LOD = Limit of Detection, LOQ = Limit of Quantification, - = not reported

Results are as submitted by participants

Table 5. Reported results (cp/cp %) and z-scores for event NK603 maize powder level 1

Maize event NK603

Laboratory number	Robust mean = 0.10 cp/cp %			
	value	LOD	LOQ	z-score
L03	1.08	0.10	0.10	4.14
L05	0.07	-	-	-0.54
L08	0.22	-	-	1.38
L09	<0.1	0.05	0.15	no z-score attributed
L10	<0.1	0.05	0.15	no z-score attributed
L13	0.10	0.05	0.15	0.01
L17	0.00	0.01	0.10	no z-score attributed
L21	<0.09	0.05	0.09	no z-score attributed
L27	0.03	-	-	-2.40
L33	0.41	-	-	2.45
L36	0.07	-	-	-0.56
L44	0.06	0.05	0.10	-0.88
L45	0.00	-	-	no z-score attributed
L46	0.10	-	-	0.01
L48	0.09	-	-	-0.21
L50	0.08	-	-	-0.38
L51	0.12	0.03	0.05	0.32
L52	0.09	0.05	0.10	-0.18
L54	0.11	0.05	0.10	0.17
L55	0.12	0.05	-	0.32
L56	0.12	-	-	0.32
L57	0.09	0.003	0.02	-0.25
L60	0.07	0.02	0.05	-0.54
L66	47.00	-	-	10.70
L71	0.11	0.02	0.06	0.17
L73	0.12	0.10	0.10	0.32
L75	0.09	0.05	0.10	-0.18
L79	0.05	0.03	0.10	-1.20
L81	0.10	-	-	0.01
L88	0.09	0.01	0.10	-0.18
L90	0.12	-	-	0.32
L91	0.13	-	-	0.46
L92	0.10	-	-	-0.08
L93	0.09	0.06	0.06	-0.19

LOD = Limit of Detection, LOQ = Limit of Quantification, - = not reported
Results are as submitted by participants

Table 6. Reported results (m/m %) and z-scores for event NK603 maize powder level 2

Maize event NK603

Laboratory number	Assigned value = 1.69 m/m % Robust mean = 1.69 m/m %				
	Value	LOD m/m	LOQ m/m	z-score[1]	z-score[2]
L01	2.62	-	-	0.76	0.76
L02	1.87	0.09	0.28	0.18	0.17
L04	1.65	0.25	0.50	-0.04	-0.05
L06	1.86	-	-	0.17	0.16
L07	1.50	0.04	0.10	-0.21	-0.21
L08	1.56	-	-	-0.14	-0.14
L09	2.00	0.10	0.10	0.29	0.29
L10	2.00	0.10	0.10	0.29	0.29
L11	1.19	0.10	0.10	-0.61	-0.61
L12	1.66	0.03	0.10	-0.03	-0.04
L14	1.90	0.50	0.50	0.20	0.20
L15	2.18	0.02	0.10	0.44	0.44
L18	1.70	-	-	0.01	0.01
L19	1.50	0.01	0.04	-0.21	-0.21
L22	1.94	0.02	0.07	0.24	0.24
L23	1.96	0.08	0.18	0.26	0.26
L24	2.40	0.00	0.00	0.61	0.60
L25	2.50	0.02	0.10	0.68	0.68
L26	1.54	0.01	-	-0.16	-0.17
L27	1.09	-	-	-0.77	-0.77
L28	1.57	0.03	0.10	-0.13	-0.13
L29	1.59	-	-	-0.11	-0.11
L30	1.81	0.01	0.01	0.12	0.11
L31	1.62	-	-	-0.07	-0.08
L32	1.27	-	-	-0.50	-0.50
L34	1.58	-	-	-0.12	-0.12
L38	2.12	-	-	0.39	0.39
L39	1.33	-	-	-0.42	-0.42
L40	1.58	-	-	-0.12	-0.12
L41	1.32	0.10	-	-0.43	-0.43
L43	1.60	-	-	-0.10	-0.10
L47	1.82	-	-	0.13	0.12
L50	1.42	0.05	0.10	-0.30	-0.31
L53	1.57	0.05	0.10	-0.13	-0.13
L58	1.50	0.01	0.10	-0.21	-0.21
L59	1.46	0.14	0.45	-0.25	-0.26
L60	1.03	0.02	0.05	-0.86	-0.86
L61	1.68	0.05	0.10	-0.01	-0.01
L62	1.47	-	-	-0.24	-0.25
L63	1.48	0.05	0.10	-0.23	-0.23
L64	1.60	0.10	0.10	-0.10	-0.10
L65	2.60	0.05	0.10	0.75	0.74
L66	2.01	0.10	0.05	0.30	0.30
L68	1.54	-	-	-0.16	-0.17
L69	1.90	0.05	0.10	0.20	0.20
L70	2.16	0.10	0.10	0.43	0.42
L72	1.90	0.01	0.07	0.20	0.20
L74	2.21	0.16	0.33	0.47	0.46
L76	1.35	0.10	0.10	-0.40	-0.40
L78	1.90	0.02	0.06	0.20	0.20
L80	4.52	0.10	0.10	1.71	1.70
L82	1.80	0.10	0.10	0.11	0.11
L83	1.75	0.02	0.09	0.06	0.06
L84	1.72	0.05	0.10	0.03	0.03
L85	1.72	-	-	0.03	0.02
L86	1.22	-	-	-0.56	-0.57
L87	1.71	-	-	0.02	0.02
L89	1.43	-	0.10	-0.29	-0.29

[1] z-score calculated on the basis of the assigned value

[2] z-score calculated on the basis of the robust mean

LOD = Limit of Detection, LOQ = Limit of Quantification, - = not reported

Results are as submitted by participants

Table 7. Reported results (cp/cp %) and z-scores for event NK603 maize powder level 2

Maize event NK603

Laboratory number	Robust mean = 1.47 cp/cp %			
	value	LOD	LOQ	z-score
L03	13.53	0.10	0.10	3.86
L05	1.18	-	-	-0.38
L08	1.76	-	-	0.31
L09	1.74	0.05	0.15	0.29
L10	1.79	0.05	0.15	0.34
L13	1.98	0.05	0.15	0.52
L17	1.71	0.01	0.10	0.26
L21	0.99	0.05	0.09	-0.69
L27	0.54	-	-	-1.73
L33	1.01	-	-	-0.66
L36	1.00	-	-	-0.67
L44	0.98	0.05	0.10	-0.70
L45	0.54	-	-	-1.74
L46	1.00	-	-	-0.67
L48	1.86	-	-	0.41
L50	1.37	0.03	0.05	-0.12
L51	1.91	0.05	0.10	0.46
L52	1.08	0.05	0.10	-0.54
L54	2.03	-	-	0.56
L55	2.02	0.05	-	0.55
L56	1.57	-	-	0.11
L57	0.86	0.00	0.02	-0.93
L60	1.38	0.02	0.05	-0.11
L66	69.00	-	-	6.69
L71	2.10	0.02	0.06	0.62
L73	2.03	0.10	0.10	0.56
L75	1.85	0.05	0.10	0.40
L79	0.70	0.03	0.10	-1.29
L81	1.90	-	-	0.45
L88	1.83	0.01	0.10	0.38
L90	1.38	-	-	-0.11
L91	1.85	-	-	0.40
L92	1.65	-	-	0.20
L93	1.27	0.06	0.06	-0.25

LOD = Limit of Detection, LOQ = Limit of Quantification, - = not reported
Results are as submitted by participants

Figure 3. z-scores for maize event NK603 on the basis of an assigned value of 0.10 m/m % for maize powder level 1

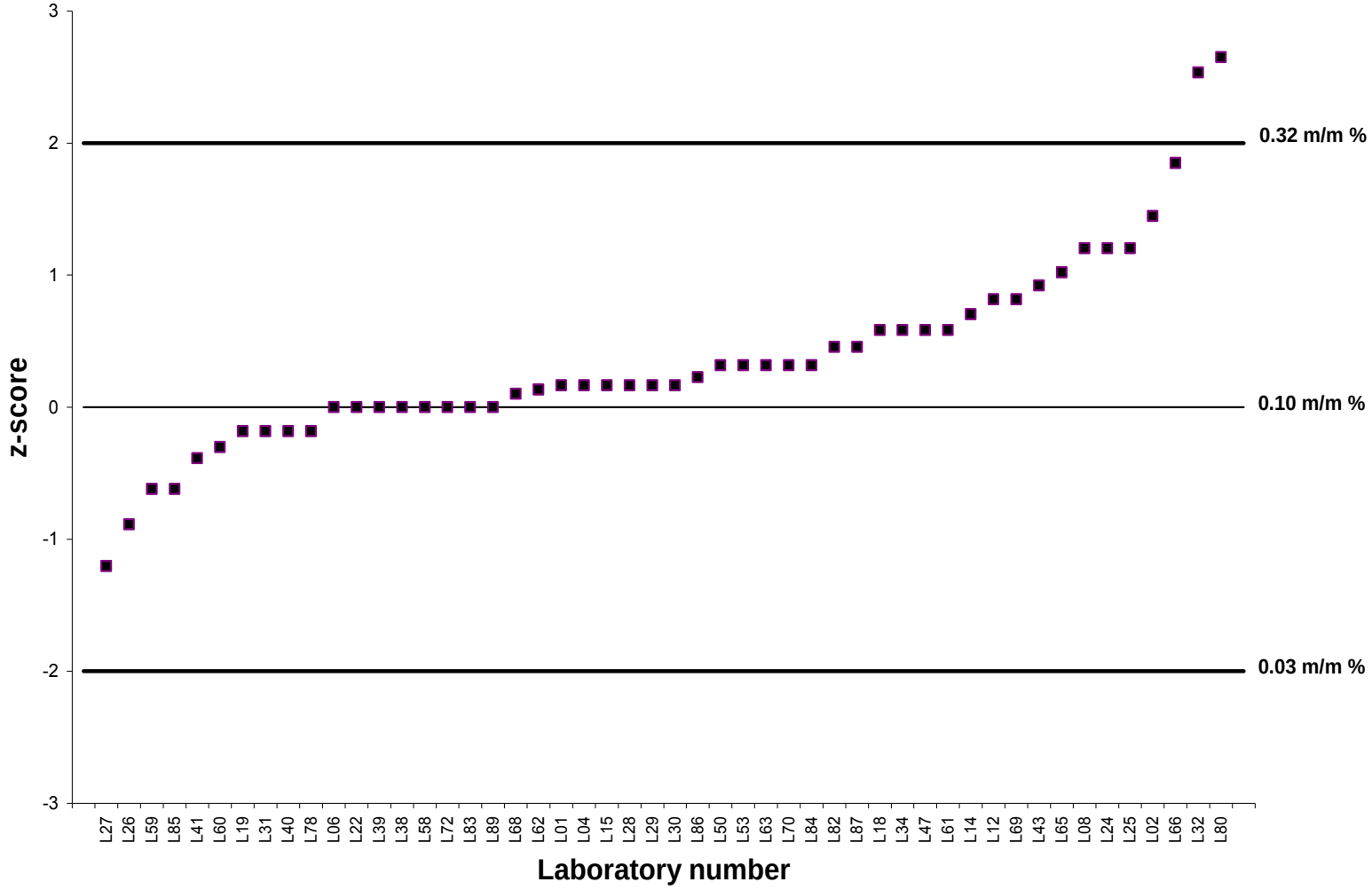


Figure 4. z-scores for maize event NK603 on the basis of a robust mean of 0.12 m/m % for maize powder level 1

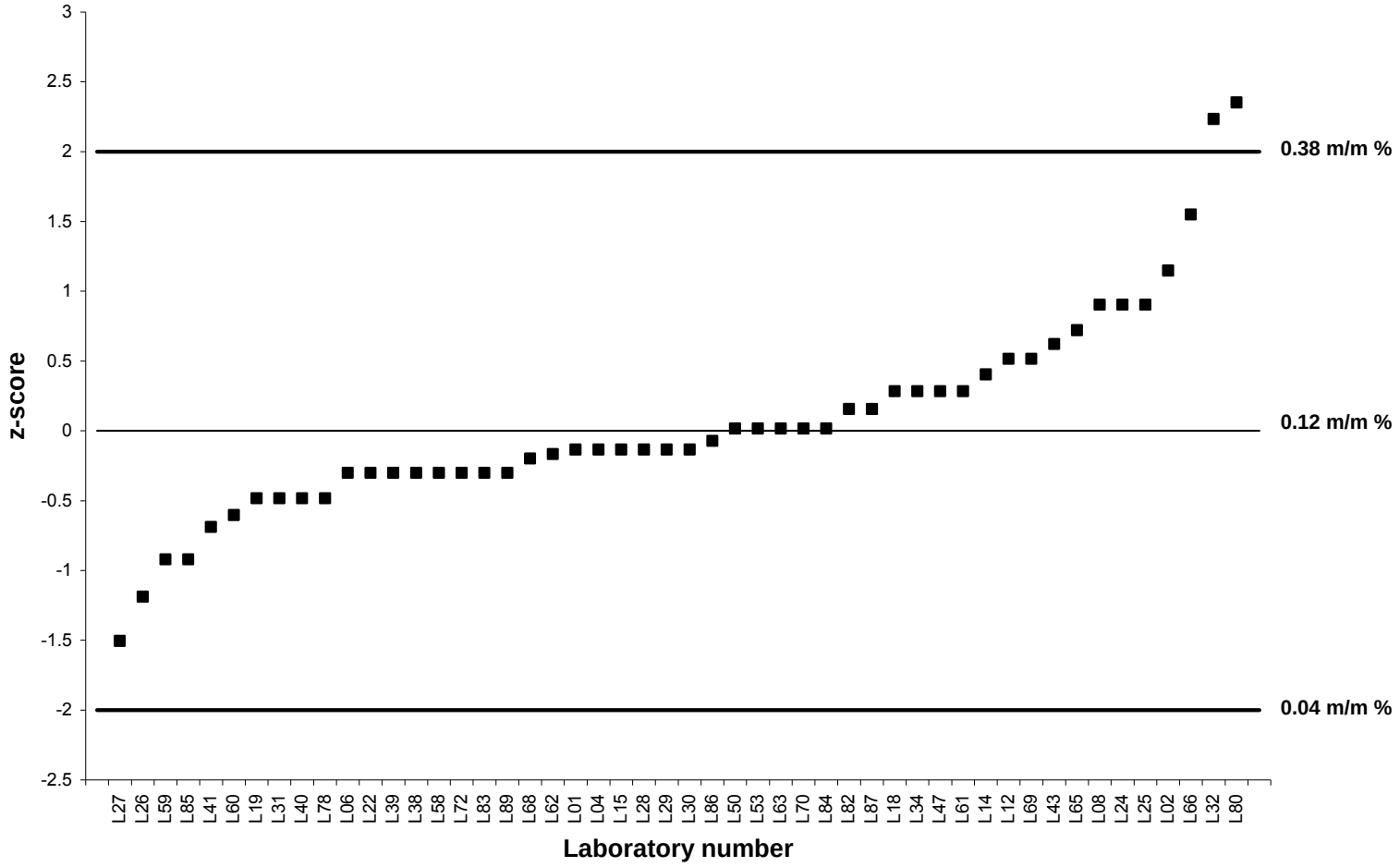


Figure 5. z-scores for maize event NK603 on the basis of a robust mean of 0.10 cp/cp % for maize powder level 1

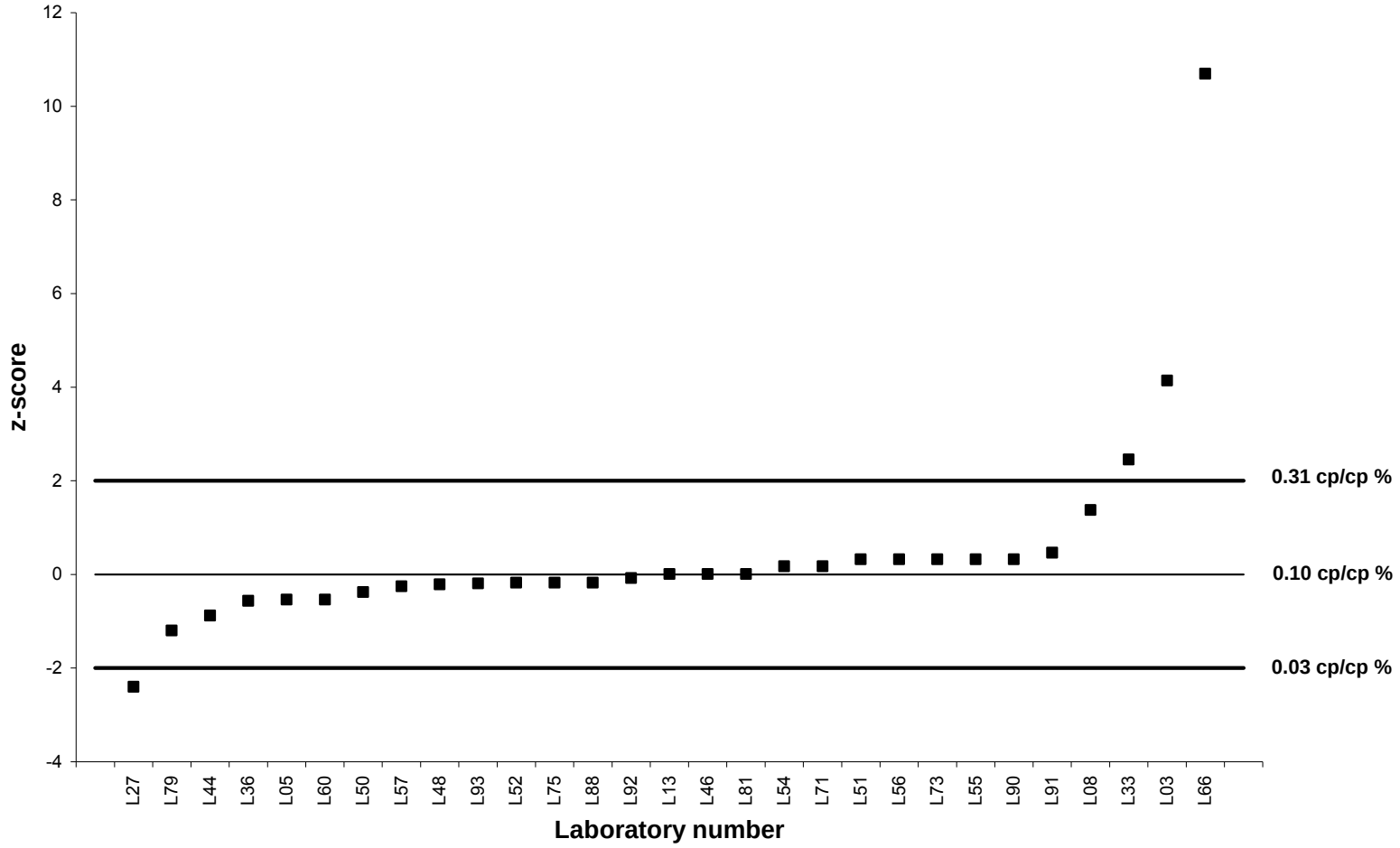


Figure 6. z-scores for maize event NK603 on the basis of an assigned value of 1.69 m/m % for maize powder level 2

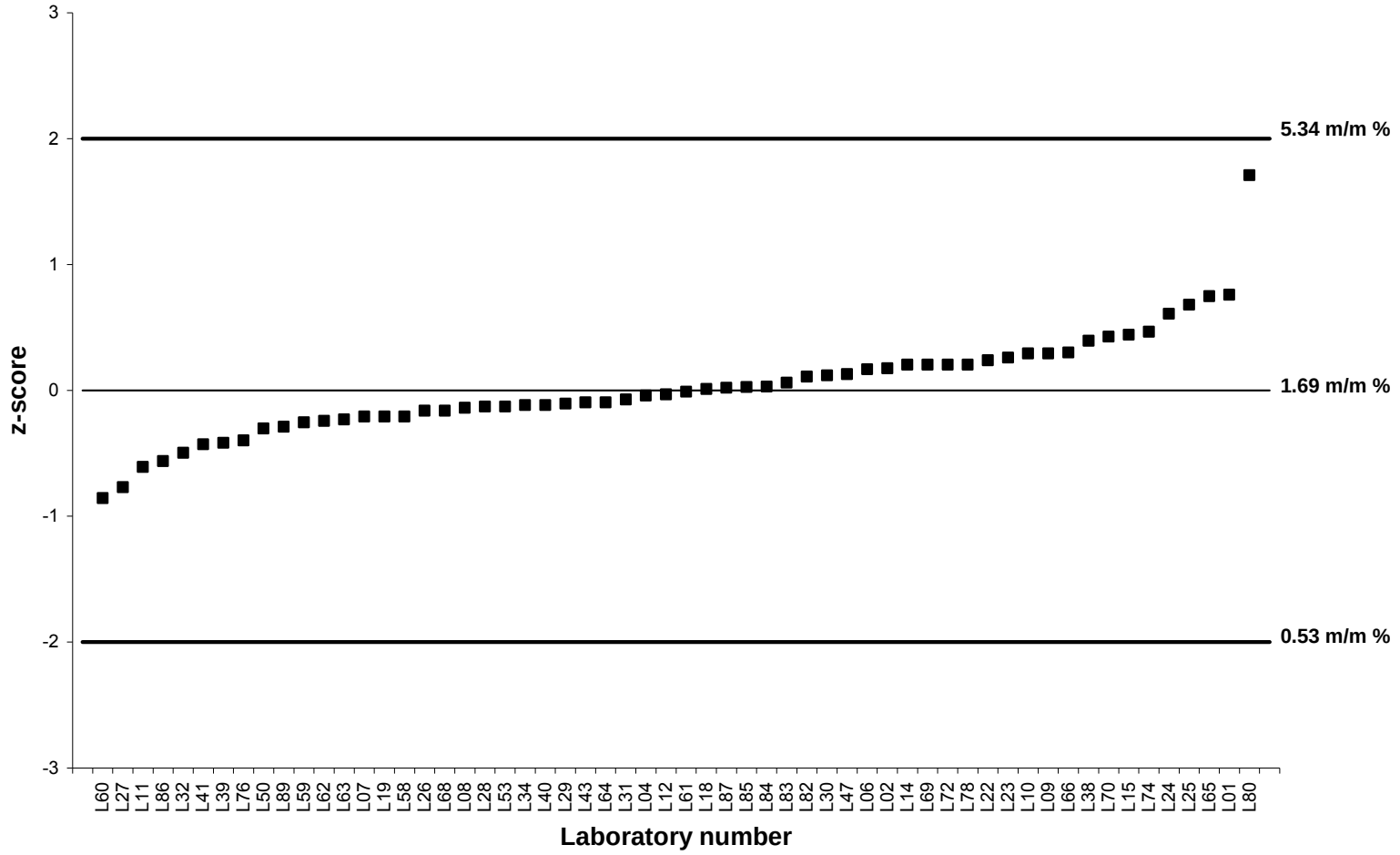


Figure 7. z-scores for maize event NK603 on the basis of a robust mean of 1.69 m/m % for maize powder level 2

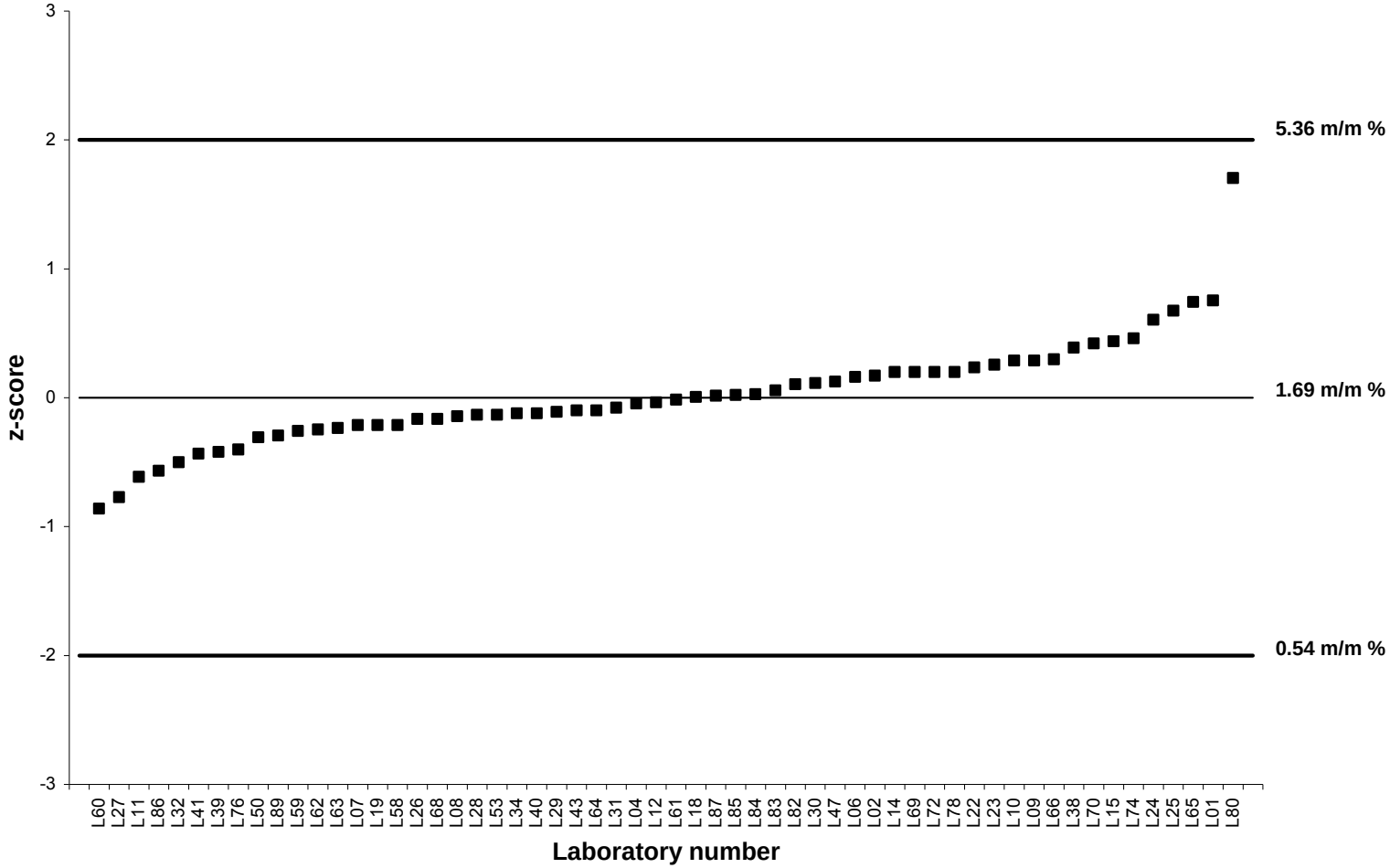
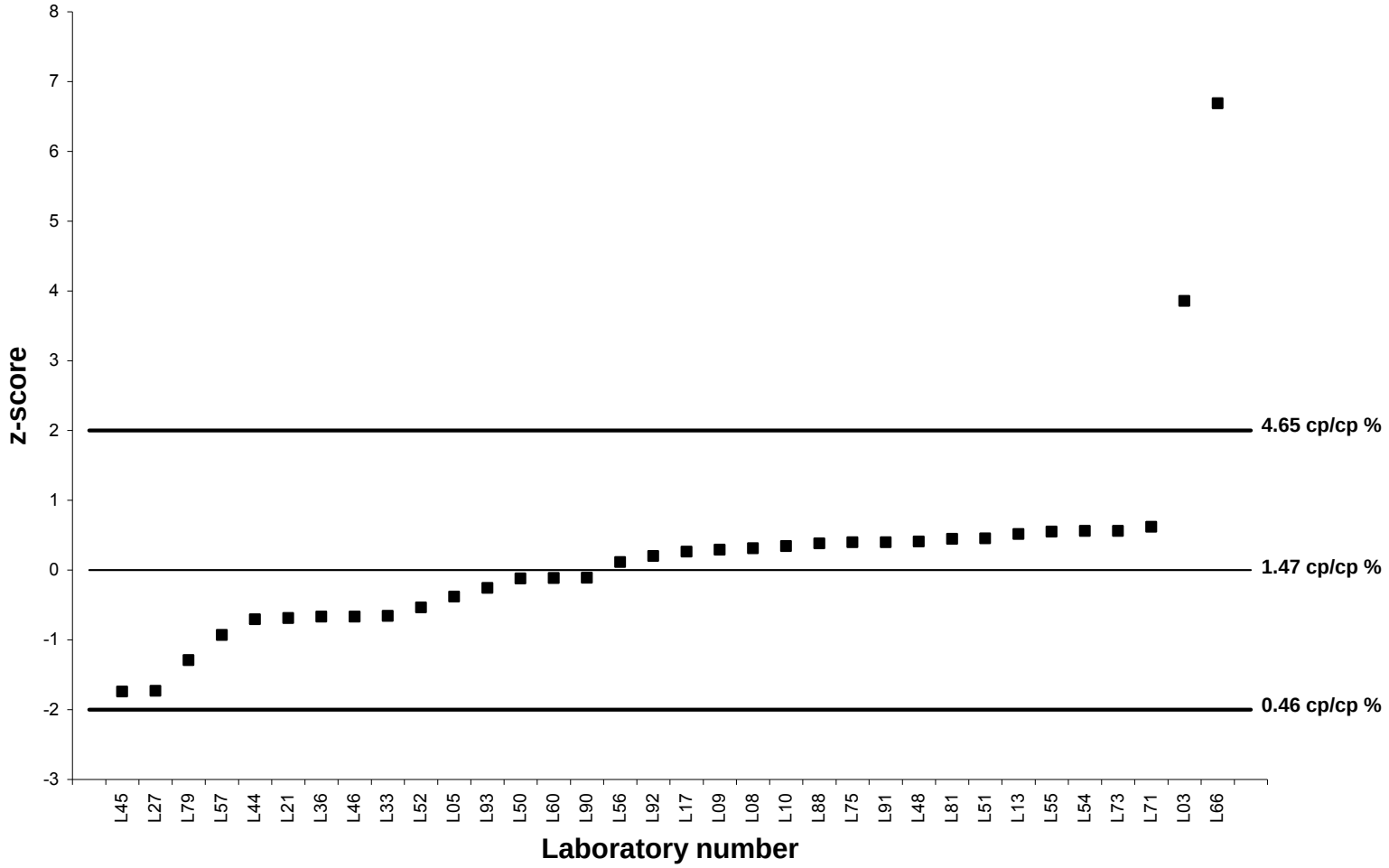


Figure 8. z-scores for maize event NK603 on the basis of a robust mean of 1.47 cp/cp % for maize powder level 2



6. Interpretation of z-scores

In general one assumes a normal distribution when calculating z-scores. In which case there is a 5 % probability that some z-scores fall outside the working range of -2 to +2 and a 0.3 % probability that some z-scores fall outside the working range of -3 to +3. A z-score outside the working range of -2 to +2 indicates that a given participant is probably not performing according to specifications but that cannot be stated with 100 % certainty. The higher the value of the standard deviation for comparative testing $\hat{\sigma}$ the more likely participants with a z-score outside the working range of -2 to +2 will be underperforming in reality. However, a higher $\hat{\sigma}$ will also increase the probability of accepting unsatisfactory measurement results. Hence a compromise should be made between the assigned $\hat{\sigma}$ value and the attempt to assess the participants' performance. In any case a z-score outside the working range of -3 to +3 will quite clearly identify an underperforming participant and will require follow-up. It should be taken into account that a well-performing laboratory has a 5 % probability of obtaining a z-score outside the working range of -2 to +2 by mere chance.
Z-scores were not determined for results reported as < value x. In which case, if the calculated value was reported as below the Limit of Quantification (LOQ) (result < x, x = LOQ), the participant could have still performed satisfactory.

7. Evaluation of results

In this first comparative testing round participants were faced with a challenge because they were asked to determine the GM content of a maize powder level 1 test item around the LOQ. Despite this challenge the outcome of this first exercise was in general very satisfactory with a share of 86-96 % and 94-100 % of participants exhibiting a z-score in the range of -2 to +2 for maize powder level 1 and level 2, respectively. As was expected, more difficulties were encountered in quantifying the 0.10 m/m % maize powder level 1 test item than in quantifying the 1.69 m/m % maize powder level 2 test item.

L20 did not report any quantitative results because of lack of instrumentation to perform quantitative analyses. The laboratory reported values < 0.1 m/m % and > 0.1 m/m % for maize powder level 1 and level 2, respectively.

For the results expressed in m/m % the assigned values determined by the RM Unit of IRMM and the consensus values determined by the EURL-GMFF through robust statistics were almost identical for level 1 (0.10 % versus 0.12 %) and were identical for level 2 (1.69 %). Hence, the number of z-scores outside the working range of -2 to +2 was identical for both approaches applied to determine a reference value. The results expressed in cp/cp % were 13-17 % lower

compared to those in m/m % (0.10 cp/cp % versus 0.12 m/m % and 1.47 cp/cp % versus 1.69 m/m % for maize powder level 1 and level 2, respectively).

For maize powder level 1 with an assigned value μ = 0.10 m/m %, nine laboratories (L07, L09, L10, L11, L20, L23, L64, L74, L76) did not report a quantitative result (Table 4), however they reported a result < value x. For laboratories L07, L09, L10, L11, L64, L74 and L76 the result reported as < value x was in agreement with their reported LOQ. L20 did not report a LOQ because no quantitative analyses were performed. Reporting inconsistencies were noted for L23. The reported LOQ was 0.18 m/m % whereas the reported result was < 0.27 m/m %. Moreover, 0.1 m/m % was the lowest concentration of the GM trait calibration curve and quantification of a 0.1 m/m % quality control material showed a coefficient of variation of 17 %. Hence, L23 should have reported a value x equal to instead of < value x for maize powder level 1. Three laboratories (L09, L10, L21) reported a result < value x whereas two laboratories (L17 and L45) reported a value equal to zero for maize powder level 1 with a robust mean of 0.10 cp/cp %. The reported results of L09, L10 and L21 were in accordance with their LOQ. L17 should have reported the result below their LOQ. The same holds true for L45. However this laboratory did not report a LOQ.

Laboratories (L05, L19, L26, L27, L31, L36, L40, L41, L44, L48, L50, L52, L57, L59, L60, L75, L78, L79, L85, L88, L92, L93) quantifying a value below 0.10 % (e.g. 0.09 %) were asked to provide information about the dynamic range of their calibration curve(s). The aim was to check if the reported result was still within their dynamic ranges. For most laboratories no discrepancies were observed. However, for L57 four out of eight PCR replicates were outside the linear working range of the calibration curve. Hence the result should have been reported as < LOQ. L40 reported a value of 0.09 m/m % whereas the lowest point of the calibration curve was 0.10 m/m %. Likewise, L40 should have reported the result as < LOQ. L59 reported a value of 0.07 m/m % which is below the LOQ of 0.45 m/m %. Hence, a value below their LOQ should have been reported as < 0.45 m/m %.

Six laboratories (L03, L27, L32, L33, L66, L80) obtained z-scores outside the working range of -2 to +2, mostly for maize powder level 1 which had a GM content around their LOQ (L03, L66 and L80). L03 and L66 obtained z-scores outside the working range of -2 to +2 for both maize powder level 1 and level 2 (Tables 5 and 7). In first instance, these laboratories were asked to provide their raw data in order to investigate the possible cause for such z-scores. In addition, L17 was asked to provide the analysis run files because it was suspected that they had swapped the values reported for maize powder level 1 and level 2, respectively. L17 and L66 did not provide any analysis run files. Some calibration curves provided by L32 and L33 showed a deviation from linearity at lower concentrations. The reported coefficients of determination (R^2) had an average value of 0.97 which is below the ENGL acceptance criterion ($R^2 \geq 0.98$)[17]. The non-linear calibration curve at lower concentrations most likely caused an overestimation of the GM content of maize powder level 1. L32 and L33 should check the DNA quality of the standard samples and test items by performing inhibition runs. In addition, the preparation of the dilution series should be investigated.

L03, L17, L27, L66, L80 were asked to repeat the experimental work. Due to import restrictions it was not possible for L32 and L33 to repeat the analyses within a time-frame of three weeks. Five new sets of test items were shipped to participants (L03, L17, L27, L66, L80) on 27 October 2010. The deadline for submission of results was 19 November 2010.

8. Performance of NRLs

Two NRLs (L35 and L37) registered for the first comparative testing round but did not report any results. L35 reported a lack of appropriate reagents for quantitative analyses. L37 stated that it had no quantitative method available for maize event NK603.

One NRL (L20) did not report any quantitative results because of lack of instrumentation to perform quantitative analyses. The laboratory reported values < 0.1 m/m % and > 0.1 m/m % for maize powder level 1 and level 2, respectively.

The first comparative testing round showed an overall positive performance of the participating NRLs. Only four NRLs (L03, L27, L66, L80) obtained z-scores outside the working range of -2 to +2. Analysing the raw data of those participants allowed identifying possible causes for these results. All four NRLs were asked to repeat the experimental work related to this first exercise. Before the shipment of a new set of test items advice was provided regarding the approach to be followed for the experimental analyses.

In the case of L03 it was suspected that a mistake occurred during the conversion of the results from the measurement unit g/kg into m/m % because the 0.1 m/m % CRM that was included as a quality control material was overestimated by a factor 10. Likewise, the reported values for maize powder level 1 and level 2 seemed to have been overestimated by a factor 10. Obviously, such a mistake would have a major impact on routine analytical results and on the decision to label a material as above the legal threshold of 0.9 %.

The z-scores outside the working range of -2 to +2 obtained by L27 were suspected to have been caused by the kit used for calibration. Since L27 is no longer an NRL, the laboratory did not repeat the experimental work.

L66 reported calibration curve slopes outside the range of ENGL acceptance criteria (-3.6 ≤ slope ≤ -3.1)[17] and was therefore advised to pay attention to this issue. However, L66 did not repeat the analyses when asked to do so. Upon the reception of a reminder message from the EURL-GMFF to submit the results related to the repetition of the experimental work the laboratory reported technical problems with the thermocycler as a cause for not reporting any results. The laboratory provided raw data from recent proficiency tests, although no information was given about the assigned value(s) and the z-score(s) obtained. L66 should re-assess their method for cp/cp % (Tables 5 and 7) calculation of the GM content since the values reported in m/m %

(Tables 4 and 6) were satisfactory. Considering the high z-scores obtained, its performance will need to be closely monitored in future comparative testing rounds.

When L80 was asked to provide the analysis run files, the laboratory immediately reported that the standards for calibration were the putative cause for their underperformance in the first comparative testing round. The analysis of their raw data confirmed their observations because different GM percentages of the standards gave almost identical Ct-values. The laboratory immediately ordered CRMs from IRMM to repeat the experimental work.

The laboratories that repeated the experimental work obtained very good results (Table 8). Z-scores were in the range of -0.30 to +0.32 which indicates a good performance.

Table 8. Repetition of experimental work: reported results (m/m % and cp/cp %) and z-scores for event NK603 maize powder level 1 and level 2

Maize event NK603			
Laboratory number			
	Assigned value = 0.10 m/m %		
	Robust mean = 0.12 m/m %		
	Value	**z-score[1]**	**z-score[2]**
L80	0.1	0.00	-0.30
	Robust mean = 0.10 cp/cp %		
	value	**z-score[2]**	
L03	0.10	0.01	
L17	0.09	-0.13	
L80	0.12	0.32	
	Assigned value = 1.69 m/m %		
	Robust mean = 1.69 m/m %		
	Value	**z-score[1]**	**z-score[2]**
L80	1.63	-0.06	-0.07
	Robust mean = 1.47 cp/cp %		
	value	**z-score[2]**	
L03	1.40	-0.08	
L17	1.66	0.21	
L80	1.42	-0.06	

[1] z-score calculated on the basis of the assigned value
[2] z-score calculated on the basis of the robust mean
Results are as submitted by participants

9. Conclusions

In this first comparative testing round participants were asked to determine the GM content in two test items containing different GM percentages of maize event NK603. Maize powder level 1 was a CRM from IRMM that was re-labelled to avoid identification by the participants as a CRM.

The second test item, maize powder level 2, was produced under validated processing conditions but never released as a CRM. The determination of a GM content around the LOQ posed a challenge to the participants.

Results could be reported either in m/m % or in cp/cp %. The majority of participants submitted the results in m/m %. The advantage of the m/m % measurement unit was that the values assigned (μ) by the test item producer (RM Unit of IRMM) could be compared with the robust means ($\hat{\mu}$) calculated by the EURL-GMFF. There was hardly any difference between the assigned value and the robust mean for maize powder level 1 (0.10 m/m % versus 0.12 m/m %) and no difference for maize powder level 2 (1.69 m/m %).

The outcome of this first exercise was in general very satisfactory with a share of 86-96 % and 94-100 % of participants exhibiting a z-score in the range of -2 to +2 for maize powder level 1 and level 2, respectively. Six laboratories obtained a z-score outside the working range of -2 to +2. The performance of these laboratories will be monitored in future comparative testing rounds. If necessary, on-site visits to those participants could be foreseen to provide assistance.

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11. Questionnaire data

The total number of answers in the questionnaire to each question does not always correspond to the total number of reported results. This is due to the fact that some questions were not answered by the participants.

1. Validation status of the DNA extraction method?	**No. of laboratories**
ISO validated	29
EURL validated	9
National reference method	2
International literature	5
In-house developed and optimised	18
Other	21

1.3. Was the DNA extraction method used within the scope of your ISO/IEC 17025 accreditation?	**No. of laboratories**

Yes	71
No	13

2. Number of replicate DNA extractions from test material?	**No. of laboratories**
1	2
2	58
3	12
4	12

3. Sample intake (in g) for the DNA extraction?	**No. of laboratories**
<0.1	1
0.1 – 0.2	26
>0.2	57

4. DNA extraction method/kit used?	**No. of laboratories**
CTAB	33
CTAB-derived	5
Dellaporta	0
Dellaporta-derived	0
Biotecon	3
DNA sorb A	0
Extragen	0
GeneScan GENE*Spin*	4
Guanidine with proteinase K	2
Macherey Nagel Nucleospin	10
Nippongene GM quicker 2	0
Promega Wizard	7
QIAmp Stool	0
Qiagen DNeasy plant mini kit	9
Qiagen QIAquick	0
R-Biopharm Rhone	0
TEPNEL kit	1
Proprietary method	0
Other	10

5. How was the clean-up of the DNA performed?	**No. of laboratories**
No DNA-clean-up	38
Ethanol precipitation	17
PEG precipitation	0

Amersham MicroSpin S300	1
Promega Wizard DNA-clean-up resin	11
Qiagen QIAquick	6
Qiagen Genomic-Tip 20/G	0
Silica	7
Propietary method	1
Other	3

6. How have you quantified the DNA?	**No. of laboratories**
Gel	1
UV spectrophotometer	35
Nanodrop	25
Fluorometer	14
Other	9

7. What was the DNA concentration (in ng/µL) of the undiluted extracted sample?	**No. of laboratories**
0-50	12
50-100	19
100-150	14
150-200	11
200-250	6
250-300	7
300-350	1
350-400	2
450-500	1
500-550	1
550-600	1
600-650	1
700-750	1
850-900	1
950-1000	2

8. Dilution buffer?	**No. of laboratories**
TE (10 mM Tris-HCl, 1 mM EDTA)	17
TE 0.1X (10 mM Tris-HCl, 0.1 mM EDTA)	14
TE low (1 mM Tris-HCl, 0.01 mM EDTA)	1
Water	43
Other	9

9. Validation status of the	**No. of laboratories**

PCR analytical method?	
ISO validated	1
EURL validated	63
National reference method	1
International literature	0
In-house developed and optimised	14
Other	5

9.3. Was the PCR analytical method used within the scope of your ISO/IEC 17025 accreditation?	**No. of laboratories**
Yes	58
No	26

10. Real-time PCR analytical method	**No. of laboratories**
Multiplex PCR	0
Singleplex PCR	84

11. Real-time PCR instrument?	**No. of laboratories**
ABI 7000	4
ABI 7300	5
ABI 7500	24
ABI 7700	4
ABI 7900HT	27
ABI StepOne & StepOnePlus real-time PCR system	1
BioRad icycler	3
Corbett Rotor-Gene 6000	2
Realplex	0
Roche LightCycler 2.0	1
Roche Lightcycler 480	4
Stratagene Mx3000/Mx3005	3
Stratagene Mx4000	0
Other	6

12. Real-time PCR plate	**No. of laboratories**
384-well plate	1
96-well plate	83

13. Real-time PCR mastermix	**No. of laboratories**
ABI TaqMan® Universal PCR master mix	52

ABI TaqMan® Universal PCR master mix, no AmpErase® UNG	5
ABI TaqMan® Fast Universal PCR master mix	1
ABI TaqMan® PCR Core Reagent Mix	0
ABI TaqMan® Gold with Buffer A	2
Agilent Technologies: Brilliant® II SYBR® Green QPCR Master Mix	0
Agilent Technologies: Brilliant® QPCR Master Mix	0
Bio-Rad: iTaq Fast Supermix With ROX	0
Bio-Rad: iQ SYBR Green Supermix	0
Eurogentec: FAST qPCR MasterMix for SYBR® Green I	0
Eurogentec: FAST qPCR MasterMix Plus	0
Promega GoTaq® qPCR master mix	0
Sigma JumpstartTM Taq ReadyMix TM	0
Proprietary real-time PCR master mix	0
Other reaction mixes	24 of which :
No information given	3
Home made	1
Qiagen	3
Roche	5
Thermo Fisher Scientific	1
Diagenode	3
Eurofins reaction mix	4
Eurogentec qPCR Mastermix	1
Fermentas	1
Ampliqon	1
Takara	1

13.1 Number of reagents involved	**No. of laboratories**
1	15
2	3
3	3
4	13
5	34
6	9
>6	6

14. Sample intake (in ng) per real-time PCR reaction	**No. of laboratories**

0-100	22
100-200	26
200-300	28
300-400	4
400-500	2
>500	2

15. Sample intake (in μL) per real-time PCR reaction	**No. of laboratories**
1	2
2	4
3	3
4	8
5	57
6-10	8
>10	2

16. Number of reactions per DNA extraction	**No. of laboratories**
1	1
2	25
3	29
4	12
5	1
6	12
>6	3

17. Number of real-time PCR cycles	**No. of laboratories**
40	4
42	1
45	66
47	1
50	9

18. Real-time PCR detection method used?	**No. of laboratories**
MGB	0
Roche probe	0
Taqman probe	83
SYBRGreen	0
Other	1

19. Real-time PCR quantification method used?	**No. of laboratories**
DNA copy number standard curve using a dilution series	33
Mass/mass standard curve using a dilution series	40
Delta Ct method	18
Other	2

20. For standard curve approach: slope - endogenous gene	**No. of laboratories**
Within Minimum Performance Requirements (MPR)[17]: -3.6 ≤ slope ≤ -3.1	59
Outside MPR	11

21. For standard curve approach: slope – GM trait gene	**No. of laboratories**
Within MPR: -3.6 ≤ slope ≤ -3.1	40
Outside MPR	30

22. For standard curve approach: R^2 coefficient - endogenous gene	**No. of laboratories**
≥0.98	62
Outside MPR	8

23. For standard curve approach: R^2 coefficient – GM trait gene	**No. of laboratories**
≥0.98	57
Outside MPR	13

24. For Delta Ct method: slope	**No. of laboratories**
Within MPR: -3.6 ≤ slope ≤ -3.1	11
Outside MPR	7

25. For Delta Ct method: R2 coefficient	**No. of laboratories**
≥0.98	16
Outside MPR	2

26. Endogenous target DNA sequences for NK603 maize?	**No. of laboratories**

Adh	45
Hmg	24
Invertase	4
Zein	3
zSSIIb	8
Other	0

27. Amplicon size (in bp) – endogenous gene	**No. of laboratories**
68	1
70	31
72	2
74	1
79	22
82	4
88	1
100	1
110	1
114	1
134	4
135	1
136	5
138	2
151	3

28. Primer and probe sequences – endogenous gene	**No. of laboratories**
F-primer: CCAGCCTCATGGCCAAAG R-primer: CCTTCTTGGCGGCTTATCTG Probe: CTTAGGGGCAGACTCCCGTGTTCCCT	32
F-primer: TTGGACTAGAAATCTCGTGCTGA R-primer: GCTACATAGGGAGCCTTGTCCT Probe: CAATCCACACAAACGCACGCGTA	21
F-primer: CGTCGTTTCCCATCTCTTCCTCCT R-primer: CCACTCCGAGACCCTCAGTC Probe: AATCAGGGCTCATTTTCTCGCTCCTCA	12
F-primer: TGGCGGACGACGACTTGT R-primer: AAAGTTTGGAGGCTGCCGT Probe: CGAGCAGACCGCCGTGTACTTCTACC	4
F-primer: CTCCCAATCCTTTGACATCTGC R-primer: TCGATTTCTCTCTTGGTGACAGG Probe: AGCAAAGTCAGAGCGCTGCAATGCA	4

F-primer: CGGTGGATGCTAAGGCTGATG R-primer: AAAGGGCCAGGTTCATTATCCTC Probe: TAAGGAGCACTCGCCGCCGCATCTG	1
F-primer: GTACCGGAACTACAAGGAGA R-primer: GAGCACGTCCTCATACAGCA Probe: CGGCATGGCGCAGGACCTCA	1
F-primer: TGCAGCAACTGTTGGCCTTAC R-primer: TGTTAGGCGTCATCATCTGTGG Probe: ATCATCACTGGCATCGTCTGAAGCGG	1
F-primer: GCATGATGCAACAAGGGC R-primer: AGGCCAACAGTTGCTGCA Probe: TTGATGGCGTGTCCGTCCCTGA	1
F-primer: CCAATCCTTTGACATCTGCTCC R-primer: GATCAGCTTTGGGTCCGGA Probe: AGCAAAGTCAGAGCGCTGCAATGCA	1
F-primer: CTCCCAATCCTTTGACATCTG R-primer: TCGATTTCTCTCTTGGTGACAGG Probe: CCGACGTGACCGACTACCACATCGA	1

29. GM trait target DNA sequence for NK603 maize?	**No. of laboratories**
35S promoter	1
CP4 EPSPS	1
NK603-specific	81
Nos terminator	1
Other	0

30. Amplicon size (in bp) – GM trait gene	**No. of laboratories**
82	2
84	2
85	1
102	1
103	2
108	69
110	1
120	1

31. Primer and probe sequences – GM trait gene	**No. of laboratories**
F-primer: ATGAATGACCTCGAGTAAGCTTGTTAA R-primer: AAGAGATAACAGGATCCACTCAAACACT	73

Probe: TGGTACCACGCGACACACTTCCACTC	
F-primer: CTCGTGCGGAGTTTTTTG	2
R-primer: GAGATGGGTTCTGCACACCA	
Probe: AGAAGTGATCAACCATGGCGAAAGTT	
F-primer: TCGGCCAGCAAGCCTTGTAG	1
R-primer: GGACTATCCCGACTCTCTTC	
Probe: GGCCGCGTTAACAAGCTTAC	
F-primer: CGACTCTCTTCTCAAGCATATGAATGA	1
R-primer: AAGCCTTGTAGCGGCCCAC	
Probe: CTCGAGTAAGCTTGTTAACGCGGCCG	
F-primer: GCCTCTGCCGACAGTGGT	1
R-primer: AAGACGTGGTTGGAACGTCTTC	
Probe: CAAAGATGGACCCCCACCCACG	

32. Which reference material was used for calibration?	**No. of laboratories**
ERM-BF415 series	13
ERM-BF415c	2
ERM-BF415d	2
ERM-BF415e	1
ERM-BF415f	57
ERM-BF415d+f	1
MON 863 X NK603 100 % (AOCS)	1
GIPSA PT SAMPLES	1
Plasmid DNA Eurofins	4
Plasmid	1

33. Which reference material was used for quality control?	**No. of laboratories**
ERM-BF415 (not specified)	10
ERM-BF415 series	6
ERM-BF415a	4
ERM-BF415b	6
ERM-BF415c	3
ERM-BF415d	16
ERM-BF415e	4
ERM-BF415f	7
ERM-BF415a+f	1
ERM-BF415b+c	2
ERM-BF415b+d	8
ERM-BF415b+e	1
ERM-BF415d+e	1

ERM-BF415d+f	1
ERM-BF415d+e+f	1
ERM-BF415b+d+e	1
ERM-BF415b+e+f	1
ERM-BF415b+d+f	1
MON 863 X NK603 100 % (AOCS)	1
pENGL-0027/04-01	1
4,5% NK603	1
Lambda DNA	1
Component of kit	1
None	5

34. Practical LOD (in %) of the GM content determination in mass/mass?	**No. of laboratories**
<0.01	1
0.01	10
0.02	7
0.03	2
0.04	1
0.05	10
0.08	1
0.09	1
0.1	10
>0.1	4

35. Practical LOD (in %) of the GM content determination in copy number ratio?	**No. of laboratories**
<0.01	5
0.01	2
0.02	2
0.03	1
0.05	10
0.06	1
0.1	2
>0.1	1

36. Practical LOQ (in %) of the GM content determination in mass/mass?	**No. of laboratories**
<0.01	1
0.01	1
0.04	1
0.05	2

0.06	1
0.07	2
0.09	1
0.1	30
>0.1	6

37. Practical LOQ (in %) of the GM content determination in copy number ratio?	**No. of laboratories**
<0.01	10
0.1	9
>0.1	3

12. Acknowledgements

We sincerely thank Roberta Brustio, Maddalena Chessa, Stéphane Cordeil, Chrystele Delobel, Sara Larcher, Encarnacion Luque-Perez, Marco Mazzara, Barbara Munaro, Steven Price, Cristian Savini, Pierluigi Tenuta, Stefania Tomasina, Guy Van den Eede of the MBG Unit and EURL-GMFF for their invaluable contributions to this first comparative testing round. A special thanks to Marko Maras who is very actively involved in the comparative testing activities. We are grateful to Philippe Corbisier, Hendrik Emons, Brigitte Fontenelle, Anne-Marie Kortekaas, Stefanie Trapmann, Ingrid Wuyts from the RM Unit of IRMM for the production of the test items and for taking care of the shipment of test items. We acknowledge Fernando Cordeiro Raposo, Beatriz De la Calle, Franz Ulberth, Inge Verbist from the FSQ Unit of IRMM for the on-line registration of participants and the management of the reported results. We are thankful to Herman Broll and Isabel Taverniers of the Advisory Board for Comparative Testing for their valuable tips to successfully complete this exercise.

The labs listed below are kindly acknowledged for their participation in this exercise.

Organisation	Department	Country
AGES - Austrian Agency for Health and Food Safety	Competence Centre Biochemistry	AT
Agricultural Institute of Slovenia	Crop and Seed Science Department	SI
Agri-Food & Veterinary Authority of Singapore	Veterinary Public Health Lab	SG
Agroscope Liebefeld-Posieux Research Station ALP	Analytics	CH
Bavarian Health and Food Safety Authority		DE
BIOMI Ltd		HU
Bundesamt für Verbraucherschutz und Lebensmittelsicherheit		DE
Center for Agricultural Technology Augustenberg	2 Ref. 24	DE
Central Agricultural Office FFSD	Laboratory for GMO Food	HU
Central Agricultural Office Food and Feed Directorate	Central Feed Investigation Lab	HU
Central Control and Testing Institute of Agriculture	Dptm. of Molecular Biology	SK
Centre Wallon de Recherches Agronomiques	Valorisation des productions	BE
Centro Nacional de Alimentacion (Agencia Española de Seguridad Alimentaria y Nutricion)	Biotechnology Unit	ES
Chemical and Veterinary Analytical Institute Muensterland-Emscher-Lippe (CVUA-MEL)		DE

Chemisches und Veterinäruntersuchungsamt Ostwestfalen-Lippe		DE
Consorcio CSIC-IRTA-UAB	SABQ	ES
Crop Research Institute	Reference Laboratory for GMO	CZ
CVUA Freiburg	Gentechnik	DE
Czech Agriculture and Food Inspection Authority	Dep. of Test.Lab.of Brno Insp.	CZ
Department of Chemistry		MY
DTU-Food, National Food Institute	Toxicology and Risk Assessment	DK
Dutch food and product safety authority		NL
Ente Nazionale Sementi Elette	Laboratorio Analisi Sementi	IT
Federal Institute for risk Assessment (BfR)	Food safety Department	DE
Federal Office of Public Health FOPH	Food Safety	CH
Fera*	Crop and Food Security	UK
Finnish Customs Laboratory	ET2	FI
GEVES	BioGEVES laboratory	FR
Hessian State Laboratory		DE
ILVO, Institute for Agricultural and Fisheries Research	Technology & Food Sciences-T&V	BE
INETI	DTIA	PT
Institut für Gesundheit und Umwelt	Gentechnik	DE
Institute for Diagnosis and Animal Health	Molecular Biology and GMO Unit	RO
Institute of Biochemistry and Biophysics PAS		PL
Institute of Chemical Technology Prague	Biochemistry and Microbiology	CZ
Institute of Food Safety, Animal Health and Environment -BIOR	Virology department	LV
Institute of Molecular Biology SAS	Laboratory of GMO	SK
Instituto Nacional de Recursos Biológicos	UIPP	PT
Instytut Zootechniki Państwowy Instytut Badawczy	Krajowe Laboratorium Pasz Prac	PL
Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana	Biotecnologie	IT
Kyung Hee University		KR
Laboratoire National de la Protection des Végétaux / National Laboratory for Plant Health		FR
Laboratoire national de Santé	Food control	LU
Laboratorio Arbitral Agroalimentario - MARM	OGM	ES
Landesamt für Umweltschutz Sachsen-Anhalt	FG 13	DE
Landesamt für Verbraucherschutz Sachsen-Anhalt	Fachbereich 3 - Lebensmittelsicherheit	DE
Landeslabor Berlin-Brandenburg	Fachbereich I-6	DE
Landeslabor Schleswig-Holstein		DE
Landesuntersuchungsamt Rheinland-Pfalz	Institut f. Lebensmittelchemie	DE
Landesuntersuchungsanstalt für das Gesundheits- und Veterinärwesen Sachsen (LUA)	Amtliche Lebensmitteluntersuch	DE
LAVES - Lebensmittelinstitut Braunschweig	Molekularbiologie	DE
LGC	Molecular and Cell Biology	UK
LSGV Saarland	Molekularbiologie	DE
Ministério da Agricultura Pecuária e Abastecimento	LANAGRO-GO	BR
Ministério da Agricultura, Pecuária e Abastecimento	LANAGRO-MG	BR
Ministério da Agricultura,Pecuária e do Abastecimento	Lab. Nacional Agropecuário	BR
Ministry of Finance, General Secretariat for Tax and Custom Issues, General Chemical State Laboratory	Food Division - Laboratory	GR
N.AG.RE.F.	G.I.L.	GR
National Center of Public Health Protection	GM food laboratory	BG
National Food Administration		SE
National Food and Veterinary Risk Assessment Institute, Laboratory Department	Molecular Biology and GMO	LT

National Institute of Biology	Biotechnology Systems Biology	SI
National Institute of Health, Istituto Superiore di Sanità	SPSVA- GMO and Mycotoxins Unit	IT
National Veterinary Research Institute		PL
Plant Breeding and Acclimatization Institute	GMO Controlling Laboratory	PL
Referral Centre for Molecular Diagnosis of Transgenic Planting Materials, National Bureau of Plant Genetic Resources	NBPGR	IN
RIKILT	NFA	NL
Science and Advice for Scottish Agriculture		UK
Scientific Institute of Public Health	SBB – GMO lab	BE
Service Commun des Laboratoires du MINEFI - Laboratoire de Strasbourg		FR
Servizio Agricola y Ganadero	Laboratorios y Estaciones Cua	CL
Shanghai Jiaotong University		CN
Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft	Geschäftsbereich 6, FB 63	DE
State General Laboratory	GMO Laboratory	CY
State Office for Agriculture, Food Safety and Fishery Mecklenburg-Western Pomerania	2/Molecular Diagnostics	DE
State Veterinary and Food Institute Dolny Kubin	Dept. of mol.-biol. analyses	SK
Tallinn University of Technology	Gene Technology	EE
The Danish Plant Directorate	Lab. for Diagnostics in Plants	DK
The National Veterinary Institute	National Feed and Food Microbiology	NO
Thüringer Landesamt für Lebensmittelsicherheit und Verbraucherschutz (TLLV)	Lab for detection of GMO in Food	DE
Thüringer State Office for Agriculture		DE
Umweltbundesamt GmbH		AT
USDA	Grain Inspection	US

*Fera also participated as NRL for Ireland

13. Annex 1: Invitation letter

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit

GM Food and Feed

Ispra, 19 January 2010
JRCI04/MBG/GVDE/ST/Ares(2010)28543

To: **All National Reference Laboratories nominated under COMMISSION REGULATION (EC) No 882/2004 of the European Parliament and of the Council of 29 April 2004 on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules.**

All National Reference Laboratories nominated under COMMISSION REGULATION (EC) No 1981/2006 of 22 December 2006 on detailed rules for the implementation of Article 32 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council as regards the Community reference laboratory for genetically modified organisms

Re: **Invitation to participate in the comparative test ILC-CRL-GMFF-CT-01/10**

Under Regulation (EC) No 882/2004 of the European Parliament and of the Council of 29 April 2004 on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules, the Community Reference Laboratory for GM Food and Feed (CRL-GMFF) shall organise comparative testing and ensure an appropriate follow-up of such comparative testing in accordance with internationally accepted protocols. Hereby, I would like to invite you to participate in the first round of comparative testing ILC-CRL-GMFF-CT-01/10. This round of comparative testing will include two test materials of a single GM event. The participant will need to quantify the GM level in each test material.

I would like to remind you that participation in comparative testing is mandatory for all NRLs nominated under Regulation (EC) No 882/2004 and Regulation (EC) No 1981/2006. Your participation is free of charge.

Comparative testing is organised by the CRL-GMFF in collaboration with the Institute for Reference Materials and Measurements (IRMM, Geel, BE). Registration for the first round of comparative testing and submission of results will be handled by IRMM. Please register electronically for the first comparative testing round using the following link: https://irmm.jrc.ec.europa.eu/ilc/ilcRegistration.do?selComparison=359

Please be aware that you need to submit multiple registration forms when you wish to apply different approaches of quantification (i.e. standard curve method, delta Ct method, different units of measurement for reporting of results).
Once you have submitted your registration electronically, print your registration form, sign it and send it to IRMM by fax or E-mail:

Fax: +32 14 571 865
Mail: JRC-IRMM-IMEP@ec.europa.eu
Cc to: mbg-comparative-testing@jrc.ec.europa.eu

Your fax/E-mail is the confirmation of your participation.

Joint Research Centre I-21027 Ispra (VA), Italy
Telephone: direct line (+39-0332) 78 5239· Telefax: (+39-0332) 78 5483.
E-mail: guy.van-den-eede@ec.europa.eu
http://ihcp.jrc.ec.europa.eu

The deadline for registration is **31 January 2010**. Samples should be shipped during the week of **8 to 12 March 2010.** The deadline for submission of results is **23 April 2010**.

If you should have any questions related to the first round of comparative testing, please contact:

Diana Charels
European Commission – Joint Research Centre
Molecular Biology and Genomics Unit – TP331
Via E. Fermi 2749
I-21027 Ispra (VA)
Phone: +39 0332 78 6518
Fax: +39 0332 78 6322
E-mail: mbg-comparative-testing@jrc.ec.europa.eu

The CRL-GMFF is looking forward to your participation.

Yours sincerely,

Guy Van den Eede
Head of Molecular Biology and Genomics Unit

Cc: SANCO: Mme Dorothée André
JRC: Mrs Hendrik Emons, Franz Uhlbert, Marco Mazzara

14. Annex 2: Accompanying letter

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit

Community Reference Laboratory
CRL
GM Food and Feed

Ispra, 3 March 2010
JRCI04/MBG/GVDE/mc/Ares(2010)111974

«Address»

Participation to ILC-CRL-GMFF-CT-01/10, a comparative testing round to quantify the GM content of maize NK603 test items

Dear «Name» «Surname»,

Thank you for participating to the ILC-CRL-GMFF-CT-01/10 comparative testing round to quantify the GM content of maize NK603 test items.

You will receive the test items shipped at room temperature via courier. The shipment will be carried out in the week of **8-12 March 2010**. On the day of the shipment we will inform you, by E-mail, about the parcel tracking number. Please make sure that someone in your laboratory is available to receive the parcel.

The parcel contains:

1. Two brown glass bottles each containing approximately 1 g of test item
2. An "Acknowledgement of Reception" form
3. This accompanying letter

Please check whether the glass bottles containing the test item remained undamaged during transport and return the "Acknowledgement of Reception" form by fax (+39 0332 789333). You should store the samples in a dark and cold place (not exceeding 18 ºC).

You should determine the GM level of NK603 in each test item received. The procedure used for quantification should resemble as closely as possible the one that you use in routine sample analyses.

The results can be reported in mass/mass % and/or copy/copy % as outlined below:

$$\text{mass/mass \%} = \frac{\text{mass GM [g]}}{\text{Total mass [g]}} \times 100\ \%$$

$$\text{copy/copy \%} = \frac{\text{GM DNA copy numbers [cp]}}{\text{Target taxon-specific DNA copy numbers [cp]}} \times 100\ \%$$

You can find the reporting website at https://irmm.jrc.ec.europa.eu/ilc/ilcReporting.do To access this webpage you need a personal password which is «PARTKEY». The system will guide you through the reporting procedure. Please enter for each test item the measurement result with its associated uncertainty.

After entering all results, please complete the questionnaire. Do not forget to save, submit and confirm when required to do so.

Directly after submitting your results and the questionnaire information on-line, you will be prompted to print the completed report form. Please sign the printed report form and return it to IRMM by fax (+32 14 571 865) or E-mail (JRC-IRMM-IMEP@ec.europa.eu). Check you results carefully before submission, since this is your final confirmation.

The deadline for submission of results is **23 April 2010**. It will not be possible to submit your results after the deadline.

Please also note that all communications during the comparative testing round should be directed to:

Diana Charels
E-mail: mbg-comparative-testing@jrc.ec.europa.eu
Phone: +39 0332 78 6518
Cc to: JRC-IRMM-IMEP@ec.europa.eu

We thank you very much for the collaboration in this comparative testing round.

Yours sincerely,

Guy Van den Eede
Head of Molecular Biology and Genomics Unit

Cc: G. Van den Eede, D. Charels, M. Mazzara

15. Annex 3: Confirmation of shipment

Dear colleague,

The present is to confirm the shipment of samples to carry out the first round of comparative testing ILC-CRL-GMFF-CT01/10. The parcels left the JRC – IRMM, Geel, by DHL express courier this morning. For your convenience, please find hereafter the corresponding airway bill number you could refer to in order to track the relevant materials on the Web:

XX XXXX XXXX

Due to the importance of this study, I would be grateful if you could acknowledge the receipt of samples and return by fax (+39 0332 789333) the letter enclosed to the package.

Should you encounter any troubles with regard to the mentioned shipment, do not hesitate to contact Brigitte Fontenelle (brigitte.fontenelle@ec.europa.eu; phone: +32 14 571 914).

Yours sincerely,
Maddalena Chessa

_____

Maria Maddalena CHESSA, Secretary of EURL-GMFF
European Commission - Joint Research Centre
Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit
Via E. Fermi, 2749
I - 21027 Ispra (VA)
Phone: + 39 0332 789379 Fax: + 39 0332 785483
E-mail: Maria-Maddalena.CHESSA@ec.europa.eu
http://www.ihcp.jrc.ec.europa.eu

16. Annex 4: Acknowledgement of receipt

FAX - Record for Quality System

JRC.I.4 -MV

Date: **R71GP6/EURL** 01/01/2009 **Acknowledgement of reception**
Page 1/1

Revision. c

From : XXXXXXXXXXXXXXXXXXX
XXXXXXXXXXXXXXXXXXX

Lab Code:
L01

To : Molecular Biology and Genomics Unit **fax:+39 0 332 78 9333**
Method Validation / EURL-GMFF
European Commission - Joint Research Centre - IHCP
21027 ISPRA (VA) Italy File nb CRL-CT-01/10

We have received the following samples	**In good condition**	**Yes**	**No**
Two brown glass bottles containing maize powder			

Comments:

Date:..... Visa:.....

Please, send this document via FAX to:
+39 0332 78 9333 the day of reception

This document is not a recognition of the quantity and/or quality of samples and reagents provided. This document will be used by EURL-GMFF only to confirm the reception of goods provided to participating laboratories in its Quality System. EURL-GMFF thanks you very much for your participation.

European Commission

EUR 24728 EN – Joint Research Centre – Institute for Health and Consumer Protection
Title: Comparative Testing Report on the Detection and Quantification of Maize Event NK603 - Comparative testing round: ILC-CRL-GMFF-CT-01/10
Author(s): D. Charels, T. Weber, M. Maras, M. Mazzara, C. Charles Delobel, E. Luque-Perez, C. Savini, G. Van den Eede
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Abstract

In the frame of Regulation (EC) No 882/2004, the European Union Reference Laboratory for Genetically Modified Food and Feed has the duty to organise comparative testing rounds and to ensure an appropriate follow-up of these activities. This report describes the outcome of the first comparative testing round ILC-CRL-GMFF-CT-01/10. Participants had to determine the GM content in two test items denoted maize powder level 1 and level 2, containing different GM percentages of maize event NK603.

This comparative testing round was organised in collaboration with the Reference Materials Unit and the Food Safety and Quality Unit of the Institute for Reference Materials and Measurements (Geel, BE). The maize event NK603 test items were produced by the Reference Materials Unit. The Food Safety and Quality Unit managed the on-line registration and submission of results.

A total of 110 laboratories were invited to participate in ILC-CRL-GMFF-CT-01/10. Five National Reference Laboratories declined participation. Ninety-three results were returned from 84 laboratories from 36 countries, of which 66 were National Reference Laboratories, seven were members of the European Network of GMO Laboratories only and 11 were laboratories from third countries. Two National Reference Laboratories, two European Network of GMO Laboratories only members and one laboratory from a third country did not submit any results.

Participants could report the results of the exercise either in mass/mass % or in copy/copy %.

The outcome of this first comparative testing round was in general positive with a share of 86-96 % and 94-100 % of participants exhibiting a z-score in the range of -2 to +2 for maize powder level 1 and level 2, respectively.

The mission of the JRC is to provide customer-driven scientific and technical support for the conception, development, implementation and monitoring of EU policies. As a service of the European Commission, the JRC functions as a reference centre of science and technology for the Union. Close to the policy-making process, it serves the common interest of the Member States, while being independent of special interests, whether private or national.

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