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Determination of GM maize event MIR604 in popcorn maize and GM soybean event A5547 in soybean powder

Report of the EURL GMFF proficiency test GMFF-23/01

EURL European Union Reference Laboratory for GM Food & Feed

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Contents

Abstract

Implementation of the European legislation on genetically modified organisms (GMOs) requires the monitoring of the presence of GMOs in food or feed by analytical tests. The analytical tests are carried out by laboratories designated for official controls by the EU Member States. To assess the uniform and reliable performance of these control laboratories proficiency tests (PTs) are organised by the European Union Reference Laboratory for Genetically Modified Food and Feed (EURL GMFF) in line with Regulation (EU) 2017/625 on official controls. This report summarises the results of the PT "GMFF-23/01" for the determination of GMOs in popcorn maize and soybean flour. Two test items consisting of popcorn maize spiked with a GM maize and ground soybean spiked with a GM soybean had to be analysed in this PT. Seventy laboratories participated to the PT round, consisting of 47 National Reference Laboratories (NRLs) from 22 EU Member States, 16 EU Official Control Laboratories (OCLs) and 7 OCLs from EU-neighbouring countries. The evaluation of the analytical performance confirms that most laboratories are able to identify and accurately quantify GMOs in food and feed samples.

Acknowledgements

BASF Belgium Coordination Center CommV is acknowledged for providing the ground A5547 soybean seeds that are used to prepare test item T2.

The first four laboratories are acknowledged for their characterisation measurements on the test items used in this PT, while the other laboratories are acknowledged for their participation to the PT round.

Organisation	**Country**
National Food Chain Safety Institute	HUNGARY
Wageningen Food Safety Research (WFSR)	NETHERLANDS
Regional Laboratory of Genetically Modified Food	POLAND
State Veterinary and Food Institute, VFI in Dolny Kubin	SLOVAKIA
Organisation	**Country**
AGES - Institute for Food Safety Vienna	AUSTRIA
Umweltbundesamt GmbH	AUSTRIA
CRA-W	BELGIUM
ILVO	BELGIUM
Sciensano	BELGIUM
Federal Institute of Agriculture Sarajevo	BOSNIA-HERZEGOVINA
Laboratory of SGS Bulgaria	BULGARIA
National Centre of Public Health and Analysis	BULGARIA
Executive Environment Agency	BULGARIA
Croatian Institute of Public Health	CROATIA
Croatian Agency for Agriculture and Food, Centre for Seed and Seedlings	CROATIA
State General Laboratory	CYPRUS
Crop Research Institute	CZECH REPUBLIC
Danish Veterinary and Food Administration	DENMARK
Finnish Customs Laboratory	FINLAND
Finnish Food Authority	FINLAND
Service Commun des Laboratoires	FRANCE
ANSES	FRANCE
BioGEVES	FRANCE
AGROLAB LUFA GmbH	GERMANY
Bavarian Health and Food Safety Authority (LGL)	GERMANY
Chemical and Veterinary Analytical Institute Münsterland-Emscher-Lippe	GERMANY
CVUA Freiburg	GERMANY
Eurofins GeneScan GmbH	GERMANY
Federal Office of Consumer Protection and Food Safety (BVL)	GERMANY
German Federal Institute for Risk Assessment	GERMANY
Institute for Hygiene and Environment	GERMANY
Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei MV (LALLF MV)	GERMANY
Landesamt für Verbraucherschutz Sachsen-Anhalt	GERMANY
Landeslabor Berlin-Brandenburg	GERMANY
Landeslabor Schleswig-Holstein	GERMANY
Landesuntersuchungsanstalt für das Gesundheits- und Veterinärwesen	GERMANY
Landwirtschaftliche Forschungs- und Untersuchungsanstalt Speyer	GERMANY
LAVES	GERMANY
LTZ Augustenberg	GERMANY
Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft	GERMANY
Thüringer Landesamt fuer Verbraucherschutz	GERMANY
General Chemical State Laboratory (GCSL)	GREECE
Eurofins Biomi Kft	HUNGARY
CREA Centro di Ricerca Difesa e Certificazione	ITALY
Istituto Zooprofilattico Sperimentale Lazio e Toscana	ITALY
Institute of Food Safety, Animal Health and Environment „BIOR"	LATVIA
National Food and Veterinary Risk Assessment Institute NFVRAI	LITHUANIA
Laboratoire National de Santé	LUXEMBOURG

Organisation	Country
Instytut Zootechniki PIB Krajowe Laboratorium Pasz Pracownia w Szczecinie	POLAND
Wojewódzki Inspektorat Weterynarii w Opolu	POLAND
National Veterinary Research institute	POLAND
Plant Breeding and Acclimatization Institute NRI	POLAND
INIAV	PORTUGAL
Institute of Diagnosis and Animal Health	ROMANIA
LCCSMS	ROMANIA
A Bio Tech lab ltd	SERBIA
SP Laboratorija a.d.	SERBIA
Central Control and Testing Institute of Agriculture (CCTIA), Bratislava	SLOVAKIA
Agrolab Ibérica SLU	SLOVENIA
Analytica Alimentaria, GmbH	SPAIN
Centro Nacional de Alimentación (CNA-AESAN OA)	SPAIN
Generalitat de Catalunya	SPAIN
Instituto de Ciencias de la Salud. Junta de Comunidades de Castilla la Mancha	SPAIN
Laboratorio Agroalimentario Gobierno de Navarra	SPAIN
Laboratorio Arbitral Agroalimentario – MAPA	SPAIN
Laboratorio Central de Veterinaria	SPAIN
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SeLyC	SPAIN
Swedish Food Agency - Livsmedelsverket	SWEDEN
Agroscope	SWITZERLAND
Federal Institute of Metrology METAS	SWITZERLAND
National Food Reference Laboratory (Tarım ve Orman Bakanlığı Ulusal Gıda Referans Laboratuvarı, Müdürlüğü)	TURKEY
Ankara Food Control Laboratory (Ankara Gıda Kontrol Laboratuvar Müdürlüğü)	TURKEY

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The report has been authorised for publication by Ursula Vincent, Head of Unit F.5.

Executive summary

The European Union Reference Laboratory for Genetically Modified Food and Feed (EURL GMFF) organised the proficiency test (PT) "GMFF-23/01" for the determination of GMOs in food and feed products to support the implementation of Regulation (EU) 2017/625 [1]. This PT was open to National Reference Laboratories (NRLs) and official control laboratories (OCLs) and was managed in line with ISO 17043:2010 [2].

Two test items were distributed to participants. Test item T1 consisted of a ground popcorn maize spiked with flour of maize event MIR604 (Unique Identifier SYN-IR6Ø4-5). Test item T2 was composed of ground soybean seeds spiked with ground seeds of GM soybean event A5547 (Unique Identifier ACS-GMØØ6-4). The EURL GMFF evaluated the homogeneity and stability of the test items. The assigned values were determined as consensus value from the results of a dedicated inter-laboratory comparison involving five selected expert laboratories. The assigned values (with expanded measurement uncertainty [k=2]) were 0.423 ± 0.096 m/m % for MIR604 and 1.944 ± 0.272 m/m % for A5547.

Seventy laboratories participated to the PT round, consisting of 47 NRLs from 22 EU Member States, 16 EU OCLs and 7 OCLs from EU-neighbouring countries. For both test items one laboratory indicated that it was out of the scope of the laboratory and therefore not analysed, hence each of the test items was analysed by 69 laboratories.

The first step in GMO analysis, following DNA extraction, is the qualitative identification of any GM event(s) present in the test items. Most of the laboratories (56 out of 69) identified the MIR604 event in T1, while 11 did not test this GM event. Similarly, 60 laboratories (out of 69) identified the A5547 event in T2, while 9 did not test for its presence.

The quantitative results reported for the GM event in T1 and T2 were evaluated using z (or z') and zeta (ζ) scores, in accordance with ISO 13528:2022 [3]. The relative standard deviation for proficiency assessment (σ_{pt}) for both GM events was set to 25 % of the respective assigned values, based on the experience acquired in previous PT rounds.

Fifty-five of the 56 laboratories reported a total of 61 quantitative results for MIR604 in T1 (some laboratories reported results obtained by qPCR and dPCR). The majority of these results (85 %) were considered satisfactory, expressed as a z' score. Similarly, 51 laboratories (out of 61) reported a total of 57 quantitative results for A5547 in T2 (some laboratories reported results obtained by qPCR and dPCR). The vast majority of the results (91 %) were considered satisfactory, expressed as a z score.

Six laboratories analysed T1 and/or T2 using both qPCR and dPCR. While most of the results were acceptable, the dPCR results were in general closer to the assigned value compared to the qPCR results.

Most participants reported a realistic expanded measurement uncertainty and coverage factor for their results. The participants were also asked to evaluate the compliance of the test items against the applicable EU legislation on GMOs. Over 92 % (T1) and 84 % (T2) of the laboratories having quantified the GM events provided a correct compliance statement for the two test items investigated.

This PT round confirms that most NRLs and OCLs are able to monitor and quantify mass fractions of GMOs in food and feed samples in the frame of Regulation (EU) 2017/625.

1 Introduction

The European Union Reference Laboratory for Genetically Modified Food and Feed (EURL GMFF), hosted by the Joint Research Centre of the European Commission, organised a proficiency testing (PT) round for the determination of the mass fractions of GM maize event MIR604 in popcorn maize and GM soybean event A5547 in soybean flour, to support Regulation (EU) 2017/625 on official controls [1].

This PT was agreed with the Directorate General for Health and Food Safety (DG SANTE) as part of the EURL GMFF annual work programme for 2023, thus complying with the mandate set in Regulation (EU) 2017/625 [1]. The PT round was open to National Reference Laboratories under Regulations (EU) 2017/625 (NRL/625) and (EU) No 120/2014 (NRL/120) [4] and, under certain conditions, also to official control laboratories (OCLs).

Two test items were prepared and dispatched to participants for analysis. Popcorn maize spiked with MIR604 maize flour (food, test item T1) was selected to resemble food products analysed by control laboratories in the EU. The second sample (feed, test item T2) consisted of ground whole soybean flour spiked with ground seed powder of A5547 soybean.

This report summarises the outcome of the PT.

2 Scope

The present PT aims to assess the performance of NRLs and OCLs in the determination of the mass fractions of GMOs in market-relevant food and feed products.

The PT was mandatory for the NRL/625, recommended for NRL/120, and open to OCLs (under certain conditions). Participants were also asked to provide a compliance statement for each test item in relation to the applicable EU Regulations (EC) No 1829/2003 [5] and (EU) No 619/2011 [6].

This PT, organised in line with ISO/IEC 17043:2010 [2], is identified as "GMFF-23/01".

3 Set up of the exercise

3.1 Quality assurance

The JRC Unit hosting the EURL GMFF is accredited according to:

- ISO/IEC 17025:2017 (certificate number: BELAC 268-TEST, flexible scope for genetically modified content in % (m/m) and % (cp/cp) in food and feed); and
- ISO/IEC 17043:2010 (certificate number: BELAC 268-PT, proficiency test provider)

The reported results were evaluated following the relevant administrative and logistic procedures.

3.2 Confidentiality

The procedures used for the organisation of PTs guarantee that the identity of the participants and the information provided by them are treated as confidential. The participants in this PT received a unique laboratory code used throughout this report. However, the laboratory codes of NRLs appointed in line with Regulation (EU) 2017/625 [1] may be disclosed to DG SANTE upon request for the purpose of an assessment of their (long-term) performance. Similarly, laboratory codes of appointed OCLs may be disclosed to their respective NRL upon request.

3.3 Time frame

Announcement (Annex 1), sent to NRLs and selected non-EU OCLs	23 March 2023
Registration deadline	19 April 2023
Sample dispatch	3 May 2023
Results deadline	16 June 2023, extended to 25 June 2023 by email announcement to all participants on 7 June 2023

3.4 Distribution

Each participant received:

- One bottle of test item T1 (ground popcorn), containing approx. 5 g of dry powder;
- One bottle of test item T2 (soybean flour), containing approx. 5 g of dry powder;
- A general "Test item accompanying letter" (Annex 2).

Samples were dispatched at room temperature. Participants were asked to check whether the bottles were damaged after transport and to store the test items in a cool place at approximately 4 °C.

3.5 Instructions to participants

Detailed instructions were given to participants in the "Instructions letter" (Annex 3), sent by email on the day of the dispatch, and providing the individual lab code to be used by every participant when submitting the results obtained.

The test items were described as two ground test materials, "*derived from imported samples that are not declared as containing GM material"*. The testing laboratories were requested to check the presence of GMOs and assess the compliance of the samples with the applicable GMO legislation (assuming that all GMO presence would be adventitious or technically unavoidable).

Participants were requested to perform the following analyses:

Test Item 1 – Popcorn maize (food):
- Verify the presence of GM maize in the sample;
- Quantify the GM event(s) identified and assess compliance of the sample.

Test Item 2 – Soybean powder (for feed):
- Verify the presence of GM soybean in the sample;
- Quantify the GM event(s) identified and assess compliance of the sample.

Participants were informed that the procedure used for the analysis should resemble as closely as possible their routine procedures for this type of matrix and GM mass fraction levels. The quantitative results had to be expressed in mass/mass %. Since the homogeneity study was performed with 200 mg sample intake for T1 and T2, the recommended minimum sample intake was set to this amount.

When submitting their results, participants were instructed (i) to select the appropriate option (e.g. "absent" (default), "present", "not tested" (for qualitative tests), or "m/m %" (when entering a quantitative value)), (ii) to report results with their measurement uncertainty and coverage factor *k*; and (iii) to select the technique used from a drop-down list.

Participants received an individual code to access the on-line reporting interface for reporting their measurement results.

Participants were also asked to fill in an online EU Survey questionnaire, accessible with a provided password. The questionnaire was designed to collect additional information related to the methods used by the laboratories when performing the measurements.

Additionally, registered participants were notified that the dual registration was enabled to allow the same laboratory to report two results for a single sample using qPCR and dPCR techniques.

4 Test items

4.1 Preparation

Test item T1 was prepared from popcorn maize and MIR604 maize. The ground popcorn was tested for the presence of traces of GM maize events with pre-spotted plates [7, 8] and no events were found. The processing of the materials was done as detailed in Table 1.

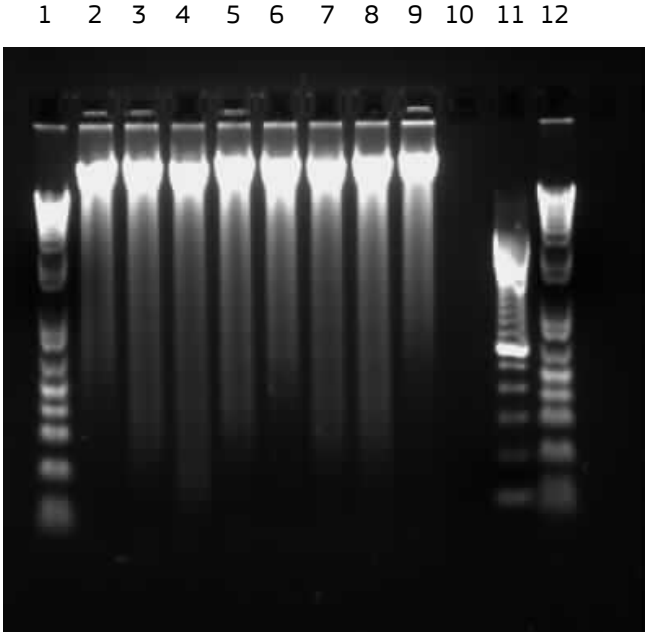
Table 1. Characteristics of the base materials used for the preparation of T1

Characteristic	Popcorn	MIR604 maize
Type of base material	Whole seed	Fine flour
Origin	Local grocery, brand Boni (BE)	100 % MIR604 flour used to prepare the ERM-BF424 series
Grinding equipment	Cryo-grinding vibrating mill	/
Mixing equipment	Turbula T2 mixer (step 1) / Dynamix CM200	
Water content in g/100 g, mean ± *U* (*k*=2), with *n*=3	2.16 ± 0.27	1.10 ± 0.12
Particle diameter in µm, mean ± *U*[1] (*k*=2), with *n*=3	123.5 ± 5.6	120.1 ± 25.7
Mass used to prepare T1 (g) – STEP 1	98.63	3.54
Mass used to prepare T1 (g) – STEP 2	613.25	101.89 g of step 1

[1] Average equivalent sphere diameter of the X_{50} size class on the cumulative volume distribution curve
k: coverage factor; *U*: expanded measurement uncertainty

The amount and the quality of the DNA extracted from the T1 material using a CTAB method (without Genomic-tip20 purification) were verified by UV spectrometry, fluorometry, and gel electrophoresis (Figure 1). A selection of DNA extracts was tested for PCR inhibition with the maize reference gene *hmg* target using four serial dilutions and passed the evaluation criteria (slope and ΔCq).

Figure 1. Agarose gel electrophoresis of genomic DNA extracted from the T1 material (lanes 2-9), lane 10 is an extraction blank. The molecular marker in the first and last lane is a 1 kb Plus DNA ladder (Invitrogen, USA), lane 11 shows the 100 bp DNA ladder (Invitrogen, USA).



The T1 mixture was manually filled using a vibrating feeder and a balance into 20 mL glass vials (ca. 5 g per vial) and closed under argon. The argon was added using a process scale freeze dryer (Epsilon 2 100D, Martin Christ). Each vial was capped and labelled with the PT identifier and a unique vial number. The vials were stored at +4 °C prior to shipment. A total of 125 vials were produced.

Test item T2 consisted of ground organic soybean, spiked with seed powder of GM soybean event A5547, received from BASF for this purpose (note that the CRM for this GM event consists of genomic DNA, which may become unstable when mixed with seed powder). BASF declared that the seeds were from homozygous A5547 plants and the purity of the seed batch was close to 100 % (this was not verified by the EURL GMFF). The A5547 powder was first cryoground, then mixed with the non-GM soybean flour (used also in previous PT rounds), and filled in 5 g portions into 20 ml vials, closed under argon. A total of 120 vials were produced. Further details on the processing can be found in Table 2.

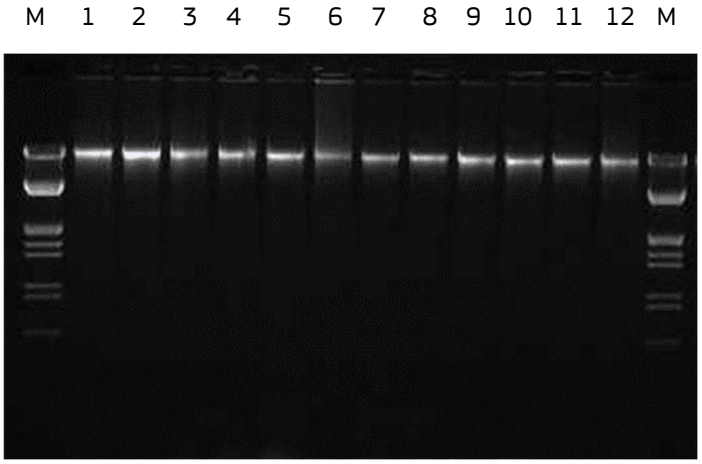
Table 2. Characteristics of the base materials used for the preparation of T2

Characteristic	Non-GM Soybean	GM Soybean A5547
Type of base material	Seeds	Coarse flour
Origin	Pit & Pit (BE) Bio–Organic Soybeans	100 % A5547 soybean
Grinding equipment	Cryo-grinding vibrating mill	Cryo-grinding vibrating mill
Mixing equipment	Turbula T2 mixer (step 1) / DynaMIX CM-200	
Water content in g/100 g, mean ± *U* (*k*=2), with *n*=3	2.93 ± 0.19	8.60 ± 0.56
Particle diameter in µm, mean ± *U*[1] (*k*=2), with *n*=3	99.9 ± 9.0	82.4 ± 14.8
Mass used to prepare T2 (g) – STEP 1	92.20	11.47
Mass used to prepare T2 (g) – STEP 2	618.11	103.44 g of step 1

[1] Average equivalent sphere diameter of the X_{50} size class on the cumulative volume distribution curve
k: coverage factor; *U*: expanded measurement uncertainty

The amount and the quality of the DNA extracted from the T2 material using a Biotecon kit (found to be suitable for this matrix) were verified by UV spectrometry, fluorometry and gel electrophoresis (Figure 2). A selection of DNA extracts were tested for inhibition with the soybean lectin reference gene *le1* target using four serial dilutions and passed the evaluation criteria (slope and ΔCq).

Figure 2. Agarose gel electrophoresis of genomic DNA extracted from the T2 material (lanes 1-12). The molecular marker in the first and last lane is the Thermo Scientific™ Lambda DNA/EcoRI plus HindIII Marker.



4.2 Homogeneity and stability

Measurements for the homogeneity and stability studies, using the corresponding event-specific detection methods (with *hmg* (79 bp) and *le1* (102 bp) as taxon-specific reference target for T1 and T2, respectively), and the statistical treatment of the data were performed by the EURL GMFF.

The assessment of **homogeneity** was performed after the processing and bottling of the test items and before distribution to the participants.

Seven bottles (of T1 and T2) were randomly selected and 3 independent replicates per bottle were used for DNA extraction (CTAB for T1 and Biotecon extraction kit for T2) and qPCR analysis. Results were evaluated according to ISO 13528:2022 [3]. The contribution from homogeneity (u_{hom}) to the standard uncertainty of the assigned value ($u(x_{pt})$) was calculated according to ISO Guide 35:2017. The T1 and T2 materials proved to be adequately homogeneous for the two GM events investigated (Annex 4.1).

The stability during dispatch conditions was assessed for T1 and T2. It was performed using an isochronous short-term stability scheme [9] involving three test samples with three replicates each (N=3, n=3) and conducted over one week at +20 °C or +40 °C. The measurements by qPCR were performed under repeatability conditions. The results revealed no significant influence of storage at +40 °C on the stability of either test item compared to storage at a reference temperature of -18 °C (data not shown). The materials were therefore dispatched at room temperature.

The **long-term stability** of the test items during the extended period covered by the PT round was also tested using qPCR, analysing the GM content in bottles (N=2, n=3) stored at the normal storage temperature of +4 °C, which has been shown to be fit for the purpose of ensuring stability in similar samples used in previous studies. Participants were also instructed to store the samples at +4 °C until analysis. The data were evaluated against the storage time and a regression line was calculated. The slope of the regression line was tested for statistical significance (i.e. increase or decrease due to storage). No significant trend was detected at a 95 % confidence level (Annex 4.2). This stability study confirmed that both test items remained adequately stable at +4 °C during the whole time period of the PT round. Hence, the uncertainty contribution to the assigned value due to instability was set to zero (u_{stab}=0) for the investigated measurands [3].

5 Assigned values and corresponding uncertainties

5.1 Assigned values

The assigned values (x_{pt}) for the mass fraction of the MIR604 event in T1 and the A5547 event in T2 were derived from measurement results obtained by qPCR in the EURL GMFF and 4 external laboratories, selected based on their performance in previous EURL GMFF PT rounds (2018-2022) and using the appropriate accredited methods. Each external laboratory was free to apply a DNA extraction method of its choice, provided that the DNA quality ($OD_{260/280}$ and $OD_{260/230}$ ratios and PCR inhibition test for an endogene target) and quantity were acceptable for qPCR.

The expert laboratories analysed two bottles of each test item, performed five independent DNA extractions from each bottle, and reported 10 results for each test item.

The assigned value for each measurand was calculated as the mean of the average results reported by the expert laboratories (Table 3), in line with ISO 13528:2022 - Section 7.6 [3]. These values are in good agreement with the nominal fractions (derived from the gravimetric preparation) of MIR604 in T1 and A5547 in T2 of 0.5 and 1.5 m/m %, respectively. However, the assigned value for A5547 in T2 is slightly larger. This may be attributed to the purity of the non-GM soybean seeds (not verified), or to a different content of lectin in the two base materials compared to the official CRM (AOCS 0707-C) (not checked). Whereas all quality parameters requested were fulfilled by all laboratories, it is noted that Lab 4 reported a lower value for A5547 compared to the other 4 laboratories; this could not be related to a particular DNA extraction method used.

Table 3. Assigned values (x_{pt}) and standard deviation for the proficiency assessment (σ_{pt}) for T1 and T2 (in m/m %).

Test item	GM event	Laboratory	DNA extraction method	Average ± *U* (*k*=2)	x_{pt}	u_{char}	u_{hom}	$u(x_{pt})$	σ_{pt}	$u(x_{pt})/\sigma_{pt}$
T1	MIR604	EURL GMFF[1]	CTAB	0.46 ± 0.18	**0.423**	0.035	0.033	**0.048**	0.116	0.45
		Lab 1	CTAB	0.38 ± 0.11						
		Lab 2	NucleoSpin Food	0.36 ± 0.07						
		Lab 3	Promega Wizard	0.54 ± 0.16						
		Lab 4	CTAB	0.37 ± 0.12						
T2	A5547	EURL GMFF[2]	Biotecon kit	1.95 ± 0.37	**1.944**	0.117	0.070	**0.136**	0.486	0.28
		Lab 1	CTAB	2.11 ± 0.59						
		Lab 2	NucleoSpin Food	2.23 ± 0.34						
		Lab 3	Promega Wizard	1.91 ± 0.57						
		Lab 4	CTAB	1.54 ± 0.47						

[1] JRC-Geel ; [2] JRC-Ispra

5.2 Associated uncertainties

The associated standard uncertainty of the assigned value ($u(x_{pt})$) was calculated following the law of uncertainty propagation, combining the standard measurement uncertainty of the characterisation (u_{char}) with the standard uncertainty contributions from homogeneity (u_{hom}) and stability (u_{stab}), in compliance with ISO 13528:2022 [3]:

$$u(x_{pt}) = \sqrt{u_{char}^2 + u_{hom}^2 + u_{stab}^2} \qquad \text{Eq. 1}$$

The uncertainty u_{char} is estimated as the standard error of the mean, according to ISO 13528:2022 [3]:

$$u_{char} = \frac{s}{\sqrt{p}} \qquad \text{Eq. 2}$$

where "s" refers to the standard deviation of the "p" dataset means and "p" refers to the number of datasets.

5.3 Metrological traceability of the assigned value

The metrological traceability to the SI of the assigned values is proven by the following facts:

- only validated methods were used during the characterisation study;
- all the values reported by the expert laboratories were traceable to the SI unit via the use of a common CRM with certified values traceable to the SI;
- satisfactory agreement of the reported results within their respective uncertainties.

5.4 Standard deviation for proficiency assessment, σ_{pt}

The relative standard deviation for PT assessment (σ_{pt}) was set to 25 % of the respective assigned values, based on the experience acquired in previous PT rounds (Table 3).

6 Scores and evaluation criteria

Laboratory competence for the (qualitative) identification of a GM event in a test item was evaluated. This information had to be selected from a drop down menu (absent [default], present, not tested or m/m %) when reporting the results through the JRC electronic platform MILC, as indicated in the instructions letter. It is expected that all laboratories who have the sample matrix and the GM event within their scope of analysis should be able to identify any GM event present in the test items.

The individual laboratory performance for the determination of the GM content was expressed in terms of *z* and ζ scores according to ISO 13528:2022 [3]:

$$z = \frac{x_i - x_{pt}}{\sigma_{pt}} \qquad \text{Eq. 3}$$

$$\zeta = \frac{x_i - x_{pt}}{\sqrt{u^2(x_i) + u^2(x_{pt})}} \qquad \text{Eq. 4}$$

where: x_i is the measurement result reported by a participant;
$u(x_i)$ is the standard measurement uncertainty reported by a participant;
x_{pt} is the assigned value;
$u(x_{pt})$ is the standard measurement uncertainty of the assigned value;
σ_{pt} is the standard deviation for proficiency test assessment.

According to ISO 13528:2022 [3], when $u(x_{pt}) > 0.3\ \sigma_{pt}$ (cf. MIR604, Table 3) the uncertainty of the assigned value ($u(x_{pt})$) should be taken into account by expanding the denominator of the *z* score and calculating the *z'* score, as follows:

$$z'_i = \frac{x_i - x_{pt}}{\sqrt{\sigma_{pt}^2 + u^2(x_{pt})}} \qquad \text{Eq. 5}$$

The interpretation of the *z* and ζ performance scores is done according to ISO 13528:2022 [3]:

|score| ≤ 2	satisfactory performance (green in Annex 5)
2 < |score| < 3	questionable performance (yellow in Annex 5)
|score| ≥ 3	unsatisfactory performance (red in Annex 5)

The ***z* scores** compare the participant's deviation from the assigned value with the standard deviation for proficiency test assessment (σ_{pt}) used as common quality criterion.

The ***ζ* scores** state whether the laboratory's result agrees with the assigned value within the respective uncertainty. The denominator is the combined uncertainty of the assigned value $u(x_{pt})$ and the measurement uncertainty as stated by the laboratory $u(x_i)$. The ζ score includes all parts of a measurement result, namely the expected value (assigned value), its measurement uncertainty in the unit of the result as well as the uncertainty of the reported values. An unsatisfactory ζ score can either be caused by an inappropriate estimation of the concentration, or of its measurement uncertainty, or both.

The standard measurement uncertainty of the laboratory $u(x_i)$ was obtained by dividing the reported expanded measurement uncertainty by the reported coverage factor, *k*. When *k* was not specified, the reported expanded measurement uncertainty was considered by the PT coordinator as the half-width of a rectangular distribution; $u(x_i)$ was then calculated by dividing this half-width by √3, as recommended by Eurachem [10].

Uncertainty estimation is not trivial, therefore an additional assessment was provided to each laboratory reporting measurement uncertainty, indicating how reasonable their measurement uncertainty estimation has been. The relative standard measurement uncertainty was calculated based on the absolute values of the assigned values [$u_{rel}(x_{pt}) = 100*(u(x_{pt})/x_{pt})$] and of the reported values [$u_{rel}(x_i) = 100*(u(x_i)/x_i)$].

The relative standard measurement uncertainty from the laboratory $u_{rel}(x_i)$ is most likely to fall in a range between a minimum and a maximum allowed uncertainty (case "a": $u_{min,rel} \leq u_{rel}(x_i) \leq u_{max,rel}$). $u_{min,rel}$ is set to the

standard uncertainties of the assigned values $u_{rel}(x_{pt})$. It is unlikely that a laboratory carrying out the analysis on a routine basis would determine the measurand with a smaller measurement uncertainty than the expert laboratories chosen to establish the assigned value (ISO 13528:2022 §7.6) or, if applicable, by formulation (ISO 13528:2022 §7.3) or than the certified measurement uncertainty associated with a certified reference material property value (ISO 13528:2022 §7.4). $u_{max,rel}$ is set to the standard deviation accepted for the PT assessment, σ_{pt} (expressed as a percentage of the assigned value). Consequently, case "a" becomes: $u_{rel}(x_{pt}) \leq u_{rel}(x_i) \leq \sigma_{pt,\%}$.

If $u_{rel}(x_i)$ is smaller than $u_{rel}(x_{pt})$ (case "b") the laboratory may have underestimated its measurement uncertainty. Such a statement has to be taken with care as each laboratory reported only measurement uncertainty, whereas the measurement uncertainty associated with the assigned value also includes contributions for homogeneity and stability of the test item. If those are large, relative measurement uncertainties smaller than $u_{rel}(x_{pt})$ are possible and plausible.

If $u_{rel}(x_i)$ is larger than $\sigma_{pt,\%}$ (case "c") the laboratory may have overestimated its measurement uncertainty. An evaluation of this statement can be made when looking at the difference between the reported value and the assigned value: if the difference is smaller than the expanded uncertainty $U(x_{pt})$ then overestimation is likely. If the difference is larger but x_i agrees with x_{pt} within their respective expanded measurement uncertainties, then the measurement uncertainty is properly assessed resulting in a satisfactory performance expressed as a ζ score, though the corresponding performance, expressed as a z score, may be questionable or unsatisfactory.

It should be understood that the reported data from participants were not $\log_{10}$-transformed prior to the performance assessment [11].

7 Evaluation of reported results

7.1 Participants

Forty-seven NRLs and 23 OCLs registered to this PT round (Table 4). NRLs responsible for managing official controls under Regulation (EU) 2017/625 (NRL/625) represented 47 % of all participants (note however, that 4 additional NRL/625 laboratories kindly agreed to perform characterisation measurements for this PT – mentioned as 'certifiers' in Table 4). All the Member States (except Malta) contributed to this PT round. Estonia and Ireland designated respectively AGES in Austria and Wageningen Food Safety Research (WFSR) in The Netherlands as their NRL for GMO analysis.

Table 4. Overview of participants to GMFF-23/01 by country and category

Country	**Participants**	**NRL/625**	**NRL/120 (and not NRL/625)**	**OCL (not NRL)**
Austria	2	2		
Belgium	3	3		
Bosnia-Herzegovina	1			1
Bulgaria	3	2		1
Croatia	2	2		
Cyprus	1	1		
Czech Republic	1	1		
Denmark	1	1		
Estonia (represented by AGES, AT)				
Finland	2	1	1	
France	3	3		
Germany	18	1	12	5
Greece	1	1		
Hungary	1	(1 certifier)		1
Ireland (represented by WFSR, NL)				
Italy	2	1	1	
Latvia	1	1		
Lithuania	1	1		
Luxembourg	1	1		
Malta	0			
Netherlands	0	(1 certifier)		
Poland	4	3 (+ 1 certifier)		1
Portugal	1	1		
Romania	2	1		1
Serbia	2			2
Slovakia	1	1 (+ 1 certifier)		
Slovenia	1	1		
Spain	10	3		7
Sweden	1	1		
Switzerland	2			2
Turkey	2			2
Total (excluding certifiers)	**70**	**33**	**14**	**23**

7.2 Qualitative results

Most of the laboratories reported qualitative results. Two NRLs indicated in the questionnaire that they did not test either T1 or T2 (matrix out of scope). Other laboratories had indicated in the MILC reporting tool that the GM event in respectively T1 or T2 was not tested.

The qualitative results are summarised in Table 5, while the individual laboratory results are presented in Annex 5.

The first step in GMO analysis of routine samples generally consists of the application of screening methods to identify the GMO elements and/or constructs that may be present or absent in the sample, thus reducing the number of event-specific methods to be applied in further analytical steps.

In **T1**, 56 laboratories identified the MIR604 event, 11 did not test for this event, one did not analyse the test item, and 2 OCLs reported that the event was "absent" (as this is the default setting, it may be a mistake). This means that almost 97 % of the laboratories who tested for the presence of the MIR604 event (56 out of 58) reported a correct qualitative result for T1. The OCLs that reported "absent" in MILC for the MIR604 event in T1 did not provide further information in the questionnaire (L51) or did not fill in the questionnaire (L66). The event-specific identification often followed the application of a varying combination of screening tests, e.g. p35S (absent), tNOS (present) and other screening targets negative (see Annex 6 for a summary of the reported screening results).

For **T2**, 60 laboratories identified the A5547 event (100 % correct), whereas 9 laboratories did not test for this event and one laboratory did not test the sample.

Therefore, all laboratories that tested the sample and corresponding GM event demonstrated their capacity to identify the correct GM event in both test matrices. Three NRL/625 and several NRL/120 and OCLs did not report the presence of the MIR604 event in T1 or the A5547 event in T2, either because the primers/probe or the CRM were not available in the laboratory, because they don't routinely analyse a food or feed matrix, because they are qualified for screening tests only or due to another reason reported in the questionnaire.

Table 5. Qualitative identification of the GM events in T1 and T2 expressed as number of laboratories

Test item and/or GM event tested?	Outcome	Detailed outcome	MIR604 in T1	A5547 in T2
Tested	Detected (D)	Only presence reported	1	3
		Quantitative result reported	55	51
		Truncated value reported	1[a]	8[b]
	Not detected (ND)	Absence reported	2	0
Not tested (NT)		Test item not tested	1 (L50)	1 (L60)
		GM event not tested	11	9
Total			70	70

[a] One laboratory reported a truncated qPCR value (< 0.9 %), but a quantitative dPCR result for the same event, therefore the truncated value is not counted up with the total number of laboratories that identified the GM event.

[b] Eight truncated values were reported by 7 laboratories; L38 reported truncated qPCR and dPCR results and L15 a truncated qPCR and quantitative dPCR result, therefore, these laboratories count only once in the total of laboratories that identified the GM event.

7.3 Quantitative results

7.3.1 Performance

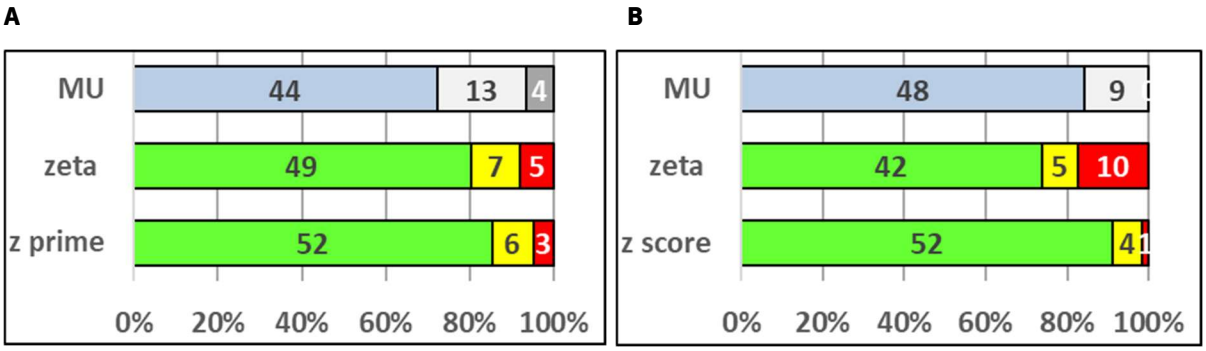
A total of 55 and 51 (out of 70) laboratories reported quantitative results for MIR604 in T1 and A5547 in T2, respectively.

The majority of participants applied real-time PCR (qPCR), while 6 laboratories reported additional dPCR results for both events, and one laboratory only a dPCR result. The option to register twice to the PT round (for reporting qPCR and dPCR results) was provided to the participants (they received a LabCode with extension "a" for qPCR and "b" for dPCR, e.g. L11a and L11b).

Laboratory performance for quantification of the GM events in T1 and T2 was expressed in terms of z (or *z'*) and ζ scores. Annex 5 presents the reported results as tables and graphs for each measurand. Satisfactory performance is highlighted in green, questionable in yellow, unsatisfactory in red. Cells were left uncoloured when the outcome could not be evaluated. The corresponding Kernel density plots (included in the main graphs) were obtained using the software available from the Statistical Subcommittee of the Analytical Methods Committee of the UK Royal Society of Chemistry [12].

Figure 3 summarises the performance scores obtained. A total of 61 and 57 results were scored for T1 and T2, respectively (including the double results reported by 6 laboratories). An overall acceptable performance (satisfactory and questionable) of 95 % (MIR604) and 98 % (A5547) is observed. Three and one unsatisfactory result(s) were reported for MIR604 and A5547, respectively. The unsatisfactory *z'* score for T1 obtained by L42 was due to a mistake in reporting the results, where the values for T1 and T2 were swapped (confirmed by L42 as soon as the preliminary report was distributed). L11 obtained for his MIR604 result by qPCR (of 0.94 m/m %) an unsatisfactory *z'* score, and a satisfactory score for his dPCR result (0.43 m/m %). The remaining unsatisfactory *z(')* scores corresponded to qPCR results overestimating the MIR604 content (L33) or underestimating the A5547 content (L45).

Figure 3. Overview of laboratory performance according to *z* and ζ scores, for the content of the event MIR604 maize in T1 (A) and A5547 soybean in T2 (B).



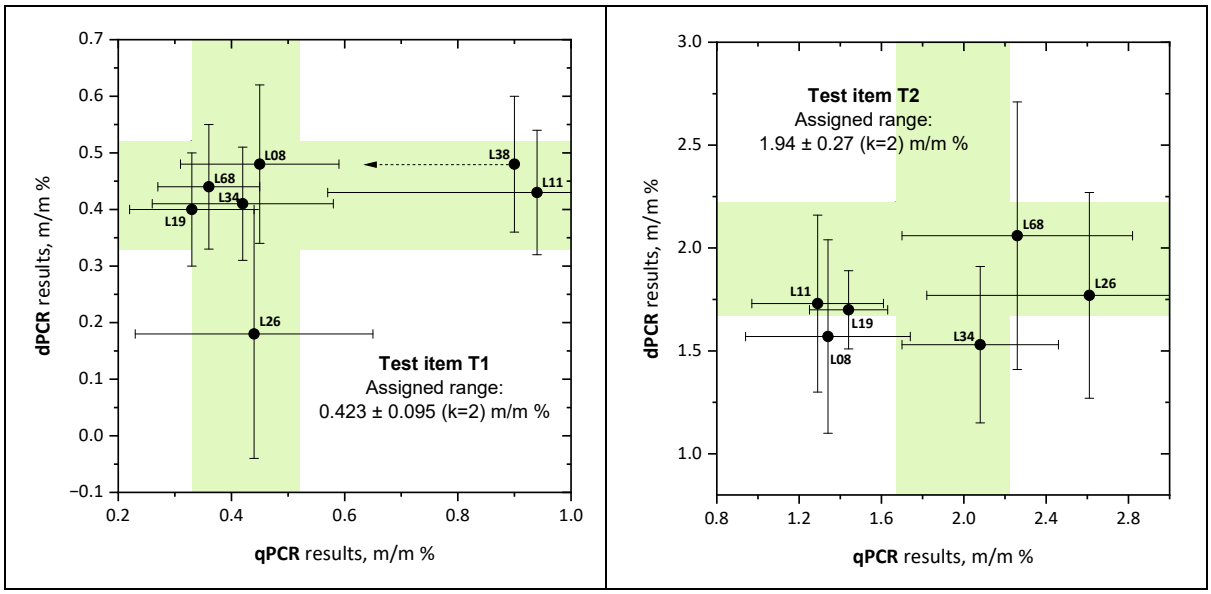
Satisfactory, questionable and unsatisfactory performance scores are indicated in green, yellow and red, respectively. Corresponding numbers of laboratories are shown in the bars. Measurement uncertainty (MU) was evaluated as follows: Case "a" (blue): $u_{rel}(x_{pt}) \leq u_{rel}(x_i) \leq \sigma_{pt,\%}$; Case "b" (light grey): $u_{rel}(x_i) < u_{rel}(x_{pt})$; Case "c" (grey): $u_{rel}(x_i) > \sigma_{pt,\%}$

7.3.2 Digital PCR results

Seven laboratories used **digital PCR** and obtained an acceptable performance scores (expressed as *z* or *z'* scores) for both test items. Despite a few exceptions, most of the reported dPCR results were closer to the assigned range than the corresponding qPCR results (Figure 4). Some observations:

- L38 only quantified T1 using dPCR and reported a truncated value (< 0.9 m/m %) for qPCR (arrow pointing left). They have applied for inclusion of the (droplet) dPCR methods under their scope of accreditation but are waiting for the finalisation of the procedure since more than a year.
- L26 mentioned in the questionnaire that none of the methods used are under their scope of accreditation. They also did not apply a conversion factor after using (chamber) dPCR to quantify MIR604 in T1. Consequently, they received a questionable *z'* score (-2.10) for this result. When applying the correct conversion factor (of 0.448), their reported value would significantly improve from 0.18 m/m % to 0.40 m/m %, much closer to their qPCR result and to the assigned value.
- L11 mentioned that the qPCR method for A5547 in T2 was not validated.

Figure 4. qPCR versus dPCR results reported by the same laboratories for T1 (left graph) and T2 (right graph). The green areas represent the assigned ranges, $x_{pt} \pm 2\ u(x_{pt})$.



7.3.3 Truncated values

One and eight truncated values were reported for MIR604 and A5547, respectively. One "less than" value was reported for MIR604 in T1, while eight "greater than" values were reported for A5547 in T2 (between > 0.01 and > 0.4), corresponding to limits of quantification (LOQ). While these values could not be included as such in the data evaluation, they were considered plausible and in line with the assigned values for the test items. Hence, the two GM events were correctly identified, but not quantified.

7.3.4 Measurement uncertainties

All laboratories having reported quantitative results, except L47 and L49 (OCL), provided expanded measurement uncertainties for both measurands (Annex 5). The missing uncertainties of these two laboratories was shown as "not provided (NP)" in the tables of Annex 5. In addition, L44 (for T1) and L62 (for T2) forgot to report the coverage factor, which was later set to 1.73 by the proficiency provider to calculate the ζ scores.

Most of the laboratories (72 % and 84 % for MIR604 and A5547, respectively) reported a realistic measurement uncertainty (Case "a" in Figure 3).

7.4 Compliance statement

Regulation (EC) No 1829/2003 [5] has established a threshold for labelling of food and feed products containing (adventitious or technically unavoidable) GM material that is authorised in the EU (0.9 m/m %). Furthermore, Regulation (EU) No 619/2011 [6] has introduced a minimum performance limit (0.1 m/m %) for detecting the accidental presence, in feed, of GM material with a pending or expired authorisation status. Compliance with these values is verified by the Member States of the European Union during the official controls on food and feed.

Laboratories were requested to provide a compliance statement for the T1 and T2 samples, in relation to the applicable EU legislation. Participants were requested to choose among five compliance statements:

CNL [Compliant: No Labelling] Compliant because no labelling required (authorised GMO mass fraction < 0.9 m/m %, if adventitious or technically unavoidable);

C<LLP [Compliant: below Low Level Presence] Compliant because GMO falling under Regulation 619/2011 was present at < 0.1 m/m % (assuming it was adventitious or technically unavoidable);

NCL [Non-Compliant: Labelling] Not compliant because the product should have been labelled (authorised GMO mass fraction > 0.9 m/m %);

NC>LLP [Non-Compliant: above Low Level Presence] Not compliant because the product contains GMOs falling under Regulation 619/2011 at a mass fraction above 0.1 m/m %;

CNC [Can Not Conclude] Compliance cannot be assessed because not all data are available.

It is important to understand that Regulation 1829/2003 and 619/2011 are mutually exclusive, i.e. a product is either:

i). Compliant to Regulation (EC) 1829/2003, when the GM event is authorised and present at a level ≤ 0.9 m/m %. When no authorised GM events are detected in the sample, this Regulation does not apply;

or

ii). Compliant to Regulation (EU) 619/2011, when the authorisation is pending or has expired, the event is included in the EU GM register related to this Regulation and it is present, in feed, at a level ≤ 0.1 m/m %. When no unauthorised GM events that are listed in the GM register under this Regulation are detected (in a feed sample), this Regulation does not apply.

The compliance statements provided for T1 and T2 samples were evaluated as summarised in Tables 6-7. Most laboratories provided a justification for their choice among the 5 compliance options. Although some testing laboratories do not usually provide such statements to their Competent Authorities when reporting their results, most European laboratories should be aware of the labelling rules in the EU and should be able to properly interpret their results.

The MIR604 event present in **T1** is authorised in the EU, therefore the reported range (result ± expanded uncertainty) is to be compared to the labelling threshold of 0.9 % (m/m) where only this Regulation applies. The following assumptions were taken into account:

- The content of MIR604 measured in T1 (0.423 m/m %) is below the threshold, hence labelling of the product is not required.
- Therefore the sample information provided (not labelled as GMO, as indicated in the instructions letter) is compliant to the applicable regulation (1829/2003), considering that the measured MIR604 traces was adventitious or technically unavoidable.
- On the basis of the measurement results obtained in the laboratory it is possible that $x - U > 0.9$ m/m %, in which case the sample should be considered not compliant to Regulation (EC) 1829/2003 because labelling would then be required (CNL).

The majority (> 92 %) of compliance statements (47 out of 51, excluding the 11 inconclusive answers) were correct (Table 6). Four laboratories additionally answered either C<LLP or NC>LLP, which is incorrect as Regulation (EU) No 619/2011 does not apply to food products and no GM events were detected that are listed in the EU GM register with pending or expired authorisation status.

Table 6. Reported compliance statements for T1 (popcorn maize)

Compliance Statement	Laboratory Measurement	Number of Laboratories	Comment
CNL - Compliant, because no labelling required	$x \pm U \leq 0.9$ m/m %	46	
	$x \pm U > 0.9$ m/m %	0	
NCL - Not compliant, should have been labelled	$x \pm U > 0.9$ m/m %	1[a]	Results switched between both test items
	$x \pm U \leq 0.9$ m/m %	0	
C<LLP - Compliant, under Regulation 619/2011 but ≤0.1 m/m %, in feed	$x \pm U \leq 0.1$ m/m %	3[b]	Wrong as this Regulation does not apply
NC>LLP - Not compliant, under Regulation 619/2011 and >0.1 m/m %, in feed	$x \pm U > 0.1$ m/m %	1[c]	Wrong as this Regulation does not apply
CNC - Cannot conclude / not quantified		11[d]	
Total no. of compliance statements		62	

[a] Assessment of L42 is correct based on the result reported, but this laboratory confirmed that they reported the results for A5547 for T1 instead of those for MIR604

[b] As the sample was popcorn maize, i.e. a food, Regulation 619/2011 does not apply. The 3 laboratories had also selected CNL which was the only correct answer.

[c] L67 explained that the MIR604 event is not authorised in the EU, which is wrong.

[d] Nine of these laboratories had not quantified the event, whereas two laboratories did quantify, but either mentioned that they are not qualified to assess compliance (L60) or did not provide a clarification for selecting this statement (L63).

For **T2** a similar assessment was made. The A5547 soybean event is authorised in the EU, therefore the reported range (result ± expanded uncertainty) is to be compared to the labelling threshold of 0.9 m/m % and only this Regulation applies. The following assumptions were taken into account:

- The content of A5547 measured in T2 (1.944 m/m %) is above the threshold.
- The lower limit of the assigned (expanded, with coverage factor 2) range for A5547 is 1.944 -0.272 = 1.672 m/m %. Taking the number of significant figures in the legislation into account, the measured value needs to be rounded to 1.7 m/m %. Hence, labelling is required for this material. As the product is not labelled as GMO (as indicated in the instructions letter for this PT) it is not compliant (NCL).
- On the basis of the measurement results obtained in the laboratory it is possible that $x - U \leq 0.9$ m/m %, in which case the sample should be considered compliant to Regulation (EC) 1829/2003 because labelling is then not required (CNL).

The majority (> 84 %) of compliance statements (43 out of 51, excluding the 15 inconclusive answers) were correct (Table 7). L31 applied an incorrect formula [$x_i - (x_i * U)$] for the compliance assessment and selected both CNL and NCL because the calculated limit for labelling was 0.93 m/m %, "*but it is up to the national monitoring officials to make the final decision on the compliance of the product*".

Two laboratories did not take the rounding of the final result into account (e.g. 0.91 or 0.93 m/m % were not rounded to 0.9 m/m % and therefore the sample was CNL). Five other laboratories additionally answered either C<LLP or NC>LLP, which is incorrect as no GM events were reported that are listed under Regulation (EU) No 619/2011.

Several laboratories were unsure about the compliance of the sample (hence reported CNC) because no quantification had been done, because a non-EU legislation applies (e.g. Turkey), or for another reason.

Table 7. Reported compliance statements for T2 (soybean flour)

Compliance Statement	Laboratory Measurement	Number of Laboratories [a]	Comment
CNL - Compliant, because no labelling required	$x \pm U \leq 0.9$ m/m %	5	
	$x \pm U > 0.9$ m/m %	1	$Xi - U$ was > 0.9, hence NCL
NCL - Not compliant, should have been labelled	$x \pm U > 0.9$ m/m %	38	
	$x \pm U \leq 0.9$ m/m %	2	$Xi - U$ = 0.9 after rounding, hence CNL
C<LLP - Compliant, under Regulation 619/2011 but ≤0.1 m/m %, in feed	$x \pm U \leq 0.1$ m/m %	3	Wrong as this Regulation does not apply
NC>LLP - Not compliant, under Regulation 619/2011 and >0.1 m/m %, in feed	$x \pm U > 0.1$ m/m %	2	Wrong as this Regulation does not apply
CNC - Cannot conclude / not quantified		15	
Total no. of compliance statements		66	

[a] Some participants provided more than one answer on compliance for the same sample

7.5 Questionnaire

The questionnaire was answered by all but five participants (L04, L32, L54, L66 and L69). As some participants provided separate answers for their qPCR and dPCR results the total number of answers received was 70 (from 65 laboratories).

The results provide valuable information about the participating laboratories, their analysis strategy and analytical approaches. Detailed information is available in Annex 6, which summarises all experimental details and comments provided by the participants. Note that not all questions were answered by all participants, therefore the total number of answers per question is not always equal to 70.

The majority of participants reported that their laboratory was accredited in accordance with **ISO/IEC 17025** for the DNA extraction method and qualitative screening methods used in the PT round, but fewer numbers were accredited for the quantitative event-specific methods. Some respondents have only accreditation for some of the methods used ("partially" accredited) or no accreditation. Among the 33 NRL/625 that answered this question, 10 and 8 are not accredited for the MIR604 or A5547 qPCR method, respectively, while 8 other NRL/625 are not accredited for both qPCR methods.

The outcome of the **screening methods** used for T1 and/or T2 are summarised. The most common screening markers were p35S, tNOS, PAT, *bar* and CTP2-CP4-EPSPS. In general, if not all GM events are tested, the laboratories usually report to their customer the results of all methods applied and occasionally indicate the events that were not tested. In such a case, 13 laboratories send the samples to another laboratory for further testing. The questionnaire also includes a question on the type of digital PCR instrument used, and whether the primers and probe concentration was modified for dPCR compared to the corresponding validated qPCR method: 7 laboratories used the same concentrations, 6 made adjustments. dPCR was preferred by these laboratories mainly because it was less affected by PCR inhibitors (12 answers) or does not need a CRM stock (7).

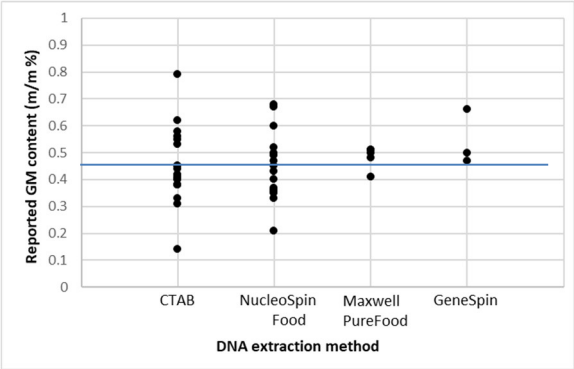
While CRMs for MIR604 are available from two producers, the legally defined CRM for MIR604 in the EU has changed on 21 January 2021 (EUR-Lex - 32021D0062 - EN - EUR-Lex (europa.eu)) from 0607-A2, available from AOCS, to ERM-BF423, produced by JRC. All but one laboratory used the correct JRC CRM for calibration. L28 used the AOCS 0706-A2 CRM and obtained a satisfactory *z* score for the result. It is not known in how far both CRMs are analytically comparable, however, it is stated on the certificates that the GM donor is the male parent in case of the JRC CRM, whereas "the MIR604 maize used in the preparation of AOCS 0607-A2 resulted from mixing three different seed materials at a ratio of 1:2:1" (GM male donor : GM female donor : non-GM seed) [https://www.aocs.org/Documents/TechnicalPDF/CRMs/0607-A2_Certificate_WEB.pdf].

Of particular interest is to verify if there was an **effect of the DNA extraction method on the GM content** reported. Different methods were used by the laboratories, mostly based on the use of (1 or 2 %) CTAB for lysis (sometimes followed by use of an automatic purification system) or using a commercial kit such as NucleoSpin Food or GeneSpin. Comparison of the reported results did not reveal an effect of the DNA

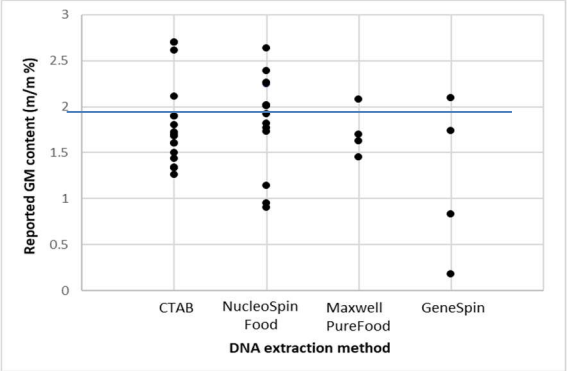
extraction method used on the results obtained in the laboratories, based on the most frequently used extraction methods (Figure 5), nor for the other methods. The results that were scored as unsatisfactory or questionable (excluding L42 who switched T1 and T2) were obtained on DNA extracted by 2 % CTAB (3 results), NucleoSpin Food (4), GeneSpin (3), SDS (1) or SpeedTools DNA extraction (1).

Figure 5. Effect of DNA extraction method used and reported GM quantity for T1 (A) and T2 (B). Horizontal line: assigned value.

A



B



8 Conclusions

The proficiency test GMFF-23/01 was organised to assess the analytical capabilities of EU NRLs and OCLs to analyse a food material (T1) and a feed material (T2) and to determine the content of MIR604 maize or A5547 soybean in these test items.

The vast majority of participants correctly identified the spiked GM events in T1 and T2 and most of these laboratories quantified these GM events. The overall performance of the participants for the determination of the content of both GM events in T1 and T2 was satisfactory (85 % for MIR604 in T1, 91 % for A5547 in T2). In addition, all reported dPCR results received an acceptable performance [$z(')$] score.

The compliance statements provided by most of the laboratories were considered in line with their reported results for T1 and T2, which shows that the control laboratories are generally competent to assess food and feed products on the EU market for the presence of GMOs and confirms their analytical capabilities to enforce the EU GMO regulations [13].

This was the first time that an inter-laboratory comparison among selected expert laboratories was set up for determination of the assigned value for the two measurands in this PT. Each of these laboratories used a DNA extraction method of their choice and all methods were applied under their accreditation scope according to ISO/IEC 17025. The assigned value was calculated as the consensus value between the results of all expert laboratories in line with ISO 13528:2022 section 7.6 [2]. This approach provides a wider basis for calculation of the assigned value compared to results from a single laboratory, as used before, and will be applied also in the upcoming PT rounds where appropriate.

References

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List of abbreviations and symbols

bp	Base pairs
dPCR	Digital Polymerase Chain Reaction
DG SANTE	Directorate General for Health and Food Safety
EU	European Union
EURL	European Union Reference Laboratory
GMFF	Genetically Modified Food and Feed
GM(O)	Genetically modified (organism)
GUM	Guide for the Expression of Uncertainty in Measurement
ISO	International Organization for Standardization
JRC	Joint Research Centre
k	Coverage factor
LOD	Limit of detection
LOQ	Limit of quantification
m/m %	GM mass fraction or mass per mass percentage
NRL	National Reference Laboratory
OCL	Official Control Laboratory
PT	Proficiency Testing
qPCR	Quantitative (real-time) Polymerase Chain Reaction
σ_{pt}	Standard deviation for proficiency test assessment
$u(x_i)$	Standard measurement uncertainty reported by participant "i"
$u(x_{pt})$	Standard uncertainty of the assigned value
u_{char}	(Standard) uncertainty contribution due to characterisation
u_{hom}	(Standard) uncertainty contribution due to inhomogeneity
u_{stab}	(Standard) uncertainty contribution due to instability
$U(x_i)$	Expanded uncertainty reported by participant "i" with the coverage factor k
$U(x_{pt})$	Expanded uncertainty of the assigned value with the coverage factor k
x_i	Mean value reported by participant "i"
x_{pt}	Assigned value
z (or z')	z (or z') score
ζ	zeta score

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Annexes

Annex 1. Invitation letter

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Directorate F – Health and Food (Geel)
Food and Feed Compliance

EURL European Union Reference Laboratory for GM Food & Feed

Geel, 23 March 2023
JRC.F.5/UV/wb/mt/ARES(2023) 23-022

FOR THE ATTENTION OF THE
NATIONAL REFERENCE LABORATORIES (NRLS) FOR GMOS
UNDER REGULATION (EU) 2017/625

Subject: Invitation to participate to the Proficiency Testing round "GMFF-23/01"

Dear National Reference Laboratory representative,

On behalf of the EURL for GM Food and Feed (EURL GMFF), we would like to invite you to participate to the proficiency test (PT) "**Determination of GM maize in popcorn (T1) and GM soybean in soybean flour (T2)**". You will receive two ground test materials. You are requested to check for the presence of GM maize (T1) or GM soybean (T2), identify and quantify the GM event(s), and assess the compliance of the samples with the applicable GMO legislation.

The PT fulfils the EURL GMFF mandate under Regulation (EU) 2017/625. Participation is free of charge.

Please register electronically by using the link below and following the instructions on screen.
https://web.jrc.ec.europa.eu/ilcRegistrationWeb/registration/registration.do?selComparison=2861

Once you have submitted your registration electronically, you will have to:

- Print your registration form, as indicated on screen
- Sign it, date it and send it to us by e-mail (JRC-EURL-GMFF-CT@ec.europa.eu)

Please register by Friday 19 April 2023.

The test items will be shipped on Wednesday 3 May 2023.

The deadline for submission of the results is Friday 16 June 2023.

The procedures used for the organisation of PTs are accredited according to ISO/IEC 17043:2010 and guarantee that the identity of the participants and the information provided by them is treated as confidential. However, the lab codes of the NRLs that have been designated in line with Regulation (EU) 2017/625 will be disclosed to DG SANTE, upon

request, for (long-term) performance assessment. Lab codes of appointed official laboratories may be disclosed to their NRL upon request.

This invitation is only sent to the NRLs. You may distribute this letter to any official laboratory within your network of official control laboratories for which you deem its participation as relevant considering all or any of the requested tasks. These laboratories will have to register for this PT using the registration details provided in this letter.

Do not hesitate to contact us (JRC-EURL-GMFF-CT@ec.europa.eu) if you have further questions.

Kind regards,

/signed electronically in Ares/

Dr. Ursula Vincent
Head of Unit

/signed electronically in Ares/

Dr. Wim Broothaerts and
Dr. Marta Cubría Radío
PT Coordinators

Annex 2. Test item accompanying letter

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Directorate F – Health and Food
Food and Feed Compliance

EURL
European Union Reference Laboratory
for GM Food & Feed

Geel, 3 May 2023

Subject: **GMFF-23/01, a proficiency test (PT) to determine the GM content in two test materials, *i.e.* popcorn maize and soybean powder**

Dear participant,

Thank you for participating to this PT round. Please find in this parcel two test materials, T1 and T2, each consisting of 5 g of ground sample.

Upon arrival, you should immediately store the samples in a fridge at ~4 °C.

Please check whether the bottles remained undamaged during transport and inform us if they arrived later than one week from the date of this letter. We will promptly replace any damaged test items.

Further instructions on this PT round, your individual lab code and the passcode for entering the results have been provided by email to the person that registered for this round.

Please, contact the functional mailbox JRC-EURL-GMFF-CT@ec.europa.eu if you have further questions.

Thank you for your collaboration.

Yours sincerely,

Wim Broothaerts
Marta Cubría Radío
PT coordinators
European Union Reference Laboratory for GM Food and Feed

Annex 3. Instructions letter

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Directorate F - Health, Consumers and Reference Materials (Geel)
Food and Feed Compliance

European Union Reference Laboratory for GM Food & Feed

Geel, 25 April 2023
JRC.F.5/WB/mt ARES(2023) 23-031

«Firstname» «Surname» (**«LCode»**)
«Organisation»
«Address»
«Zip» «Town»
«Country»

Reporting website	https://web.jrc.ec.europa.eu/ilcReportingWeb.
EU login	For help, see the Participant's guidelines
Password for reporting	«Part_key»
Questionnaire	https://ec.europa.eu/eusurvey/runner/GMFF2301
Password	GMFF2301

Subject: **Instructions for GMFF-23/01, a proficiency test (PT) to determine the GM content in two test materials, *i.e.* popcorn maize and soybean powder**

Dear Dr «Surname»,

Thank you for participating to GMFF-23/01. In one of the following days you should receive two test materials, T1 and T2, containing 5 g (dry) of ground sample, sent at ambient temperature. **The vials should be stored in a fridge at approximately 4 ºC.**

The two ground test materials are "*derived from imported samples that are not declared as containing GM material*". The testing laboratories are requested to check the presence of GMOs and assess the compliance of the samples with the applicable GMO legislation (assuming that all GMO presence would be adventitious or technically unavoidable).

Tasks
Test Item 1 – Popcorn maize (food) (5 g dry weight):
- Verify the presence of GM maize in this sample;
- Quantify the GM event(s) identified and assess compliance of the sample.

Test Item 2 - Soybean powder (for feed) (5 g dry weight):
- Verify the presence of GM soybean in this sample;
- Quantify the GM event(s) identified and assess compliance of the sample.

Participants are requested to apply their routine approaches for GMO testing. It is recommended to use a **minimum sample intake of 200 mg for your DNA extractions**, as homogeneity of the test items has been demonstrated using this amount of sample.

When **reporting your results**:

- The default setting indicates “absent” for all GM events; please change this into m/m % if reporting a quantitative result, or to “present” or “not tested” for reporting qualitative results; make sure you do this for all GM events indicated, as these results will be evaluated in the report (e.g. if you indicated “absent” for an event that was actually present, the PT report will indicate that you failed to detect the event);
- Select the “=” (default) or “<” or “>” signs for reporting values;
- Report results with their expanded uncertainty (*U*) and coverage factor *k* (mandatory for the submission);
- Do not forget to select the technique used (default is “no technique”).

Do not forget to click the "validate and save" button and the "**Submit my results**" button. Check your results carefully before submission, since this is your final confirmation. After submitting your results on-line, you should **sign the completed report form and send a pdf copy to the EURL GMFF by e-mail** as a formal validation of the data introduced through MILC. Save a copy of this form for your own records.

After submission of your quantitative results, please go to the weblink https://ec.europa.eu/eusurvey/runner/GMFF2301, enter the password (see box below address line), and answer the questions of the survey. This survey includes questions on the analytical approaches used, and a statement on compliance to EU legislation. Submit your answers to the survey on-line (no need to send them by e-mail).

Keep in mind that collusion is contrary to professional scientific conduct and serves only to nullify the benefits of proficiency tests to customers, accreditation bodies and analysts alike. Be aware of the existence of an appeal procedure in case you disagree with your scores.

The deadline for submission of the results and the questionnaire is Friday 16 June 2023. It will not be possible to submit your results after the deadline.

The EURL GMFF will analyse all data received and publish a report indicating the performance of your laboratory for the identification and quantification of the GM events. You will receive a copy of the report by e-mail. In case of an unsatisfactory performance, the NRL participants will be requested to fill in a form indicating the root-cause analysis and providing evidence demonstrating the effectiveness of the correction actions implemented. Further support may be provided in order to understand the problem and improve the analytical performance of your laboratory.

You should keep the test items at approximately 4 °C in order to voluntary repeat the analysis in case of an unsatisfactory performance. Please, dispose the test items thereafter.

Thank you for the collaboration in this PT. Please, contact the functional mailbox JRC-EURL-GMFF-CT@ec.europa.eu for all issues related to this PT round.

Yours sincerely,

e-signed

Marta Cubría Radío
Wim Broothaerts

PT coordinators

European Union Reference Laboratory for GM Food and Feed

Annex 4. Homogeneity and stability results

4.1 Homogeneity

Homogeneity of MIR604 maize in T1 (qPCR)

Bottle	Replicate 1	Replicate 2	Replicate 3
11	0.42	0.27	0.52
15	0.51	0.60	0.35
30	0.45	0.49	0.53
43	0.54	0.52	0.44
57	0.35	0.61	0.41
81	0.57	0.43	0.43
100	0.30	0.48	0.44
Mean	0.46		
s_x	0.04		
s_w	0.10		
S_s	0		
u^*	0.04		
σ_{pt}	0.116		
$0.3 * \sigma_{pt}$	0.035		
$S_s \leq 0.3^* \sigma_{pt}$	YES		
Assessment	Passed		

Homogeneity of A5547 soybean in T2 (qPCR)

Bottle	Replicate 1	Replicate 2	Replicate 3
11	1.86	2.04	2.34
15	2.05	1.93	1.80
30	1.86	2.06	1.81
43	1.78	2.18	1.79
57	2.39	1.79	1.87
81	2.05	2.01	1.81
100	1.78	1.93	1.79
Mean	1.95		
s_x	0.08		
s_w	0.20		
S_s	0		
u^*	0.07		
σ_{pt}	0.486		
$0.3 * \sigma_{pt}$	0.146		
$S_s \leq 0.3^* \sigma_{pt}$	YES		
Assessment	Passed		

Where: σ_{pt} is the standard deviation for the PT assessment,
s_x is the standard deviation of the sample averages,
s_w is the within-sample standard deviation,
s_s is the between-sample standard deviation,
u^* is the conservative value for the uncertainty associated with heterogeneity, as defined in ISO Guide 35 [14].

All values are in m/m %.

4.2 Stability

In the table below, the stability was assessed according to ISO 13528:2022 § B.5 [3].

Stability MIR604 maize in T1 (qPCR) (all values are in m/m %)

Weeks	Bottle no.	Replicate 1	Replicate 2	Replicate 3	Average
0	11	0.42	0.27	0.52	0.45
	15	0.51	0.60	0.35	
25	37	0.45	0.48	0.60	0.40
	102	0.26	0.30	0.28	

Slope ± 2 $SE_{(slope)}$ = -0.0020 ± 2 * 0.0030

Stability: passed

Stability A5547 soybean in T2 (qPCR) (all values are in m/m %)

Weeks	Bottle no.	Replicate 1	Replicate 2	Replicate 3	Average
0	59	2.16	1.66	1.52	1.70
	105	1.62	1.79	1.45	
25	37	1.60	2.01	1.99	1.81
	102	1.79	1.66	1.83	

Slope ± 2 $SE_{(slope)}$ = 0.0045 ± 2 * 0.0050

Stability: passed

Annex 5. Results and laboratory performance

MIR604 maize in T1

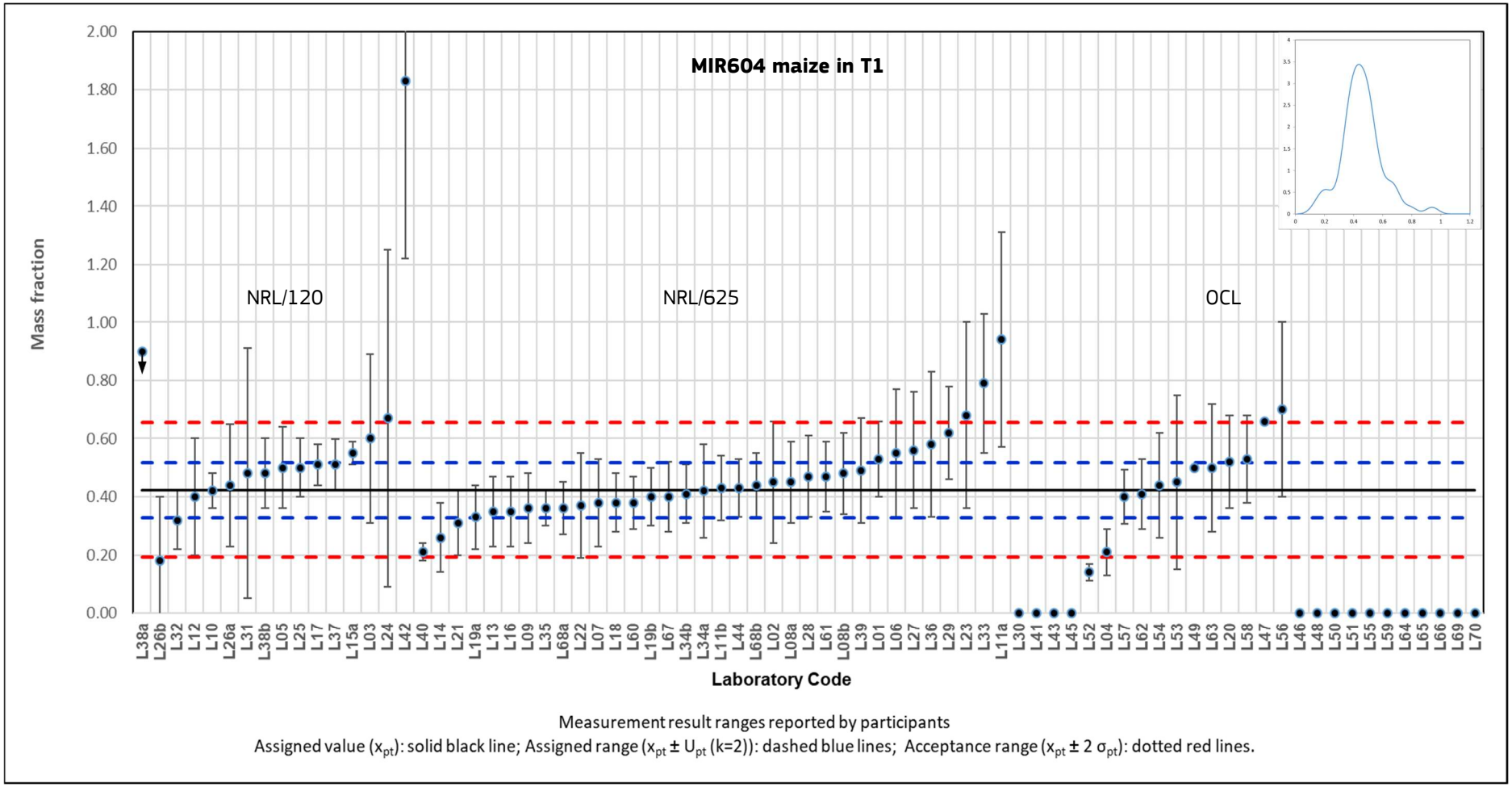
- ID = GM event identification (D = detected, ND = not detected, NT = not tested)
- The PT coordinator set the measurement uncertainty $u(x_i)$ to zero when no expanded uncertainty was reported
- The PT coordinator set k = 1.73 when no coverage factor (k) was reported
- Performance scores (z' and ζ): satisfactory, questionable, unsatisfactory
- Measurement uncertainty (MU): a: $u_{rel}(x_{pt}) \leq u_{rel}(x_i) \leq \sigma_{pt,\%}$; b: $u_{rel}(x_i) < u_{rel}(x_{pt})$; c: $u_{rel}(x_i) > \sigma_{pt,\%}$; NP = not provided

Evaluation parameters: x_{pt} = 0.423 ; $u(x_{pt})$ = 0.048 ; σ_{pt} = 0.116 (all values in m/m %)

LabType	LabCode	ID	x_i	±	k	Technique	z' score	ζ score	MU
NRL/625	L01	D	0.53	0.13	2	Real-time PCR	0.92	1.32	a
NRL/625	L02	D	0.45	0.21	2	Real-time PCR	0.23	0.23	a
NRL/120	L03	D	0.6	0.29	2.11	Real-time PCR	1.52	1.21	a
OCL	L04	D	0.21	0.08	2	Real-time PCR	-1.84	-3.43	a
NRL/120	L05	D	0.5	0.14	2	Real-time PCR	0.66	0.91	a
NRL/625	L06	D	0.55	0.22	2	Real-time PCR	1.09	1.06	a
NRL/625	L07	D	0.38	0.15	2	Real-time PCR	-0.37	-0.49	a
NRL/625	L08a	D	0.45	0.14	2	Real-time PCR	0.23	0.31	a
NRL/625	L08b	D	0.48	0.14	2	dPCR	0.49	0.67	a
NRL/625	L09	D	0.36	0.12	2	Real-time PCR	-0.55	-0.83	a
NRL/120	L10	D	0.42	0.06	2	dPCR	-0.03	-0.06	b
NRL/625	L11a	D	0.94	0.37	2	Real-time PCR	4.45	2.70	a
NRL/625	L11b	D	0.43	0.11	2	dPCR	0.06	0.09	a
NRL/120	L12	D	0.4	0.2	2	dPCR	-0.20	-0.21	a
NRL/625	L13	D	0.35	0.12	2	Real-time PCR	-0.63	-0.96	a
NRL/625	L14	D	0.26	0.12	2	Real-time PCR	-1.41	-2.13	a
NRL/120	L15a	D	0.55	0.04	2	Real-time PCR	1.09	2.45	b
NRL/625	L16	D	0.35	0.12	2	Real-time PCR	-0.63	-0.96	a
NRL/120	L17	D	0.51	0.07	2	Real-time PCR	0.75	1.47	b
NRL/625	L18	D	0.38	0.1	2	Real-time PCR	-0.37	-0.63	a
NRL/625	L19a	D	0.33	0.11	2	Real-time PCR	-0.80	-1.28	a
NRL/625	L19b	D	0.4	0.1	2.78	dPCR	-0.20	-0.39	b
OCL	L20	D	0.52	0.16	2	dPCR	0.83	1.04	a
NRL/625	L21	D	0.31	0.11	2	Real-time PCR	-0.98	-1.56	a
NRL/625	L22	D*	0.37	0.18	2	Real-time PCR	-0.46	-0.52	a
NRL/625	L23	D	0.68	0.32	2	Real-time PCR	2.21	1.54	a
NRL/120	L24	D	0.67	0.58	2	Real-time PCR	2.13	0.84	c
NRL/120	L25	D	0.5	0.1	2	dPCR	0.66	1.11	b
NRL/120	L26a	D	0.44	0.21	2	Real-time PCR	0.14	0.14	a
NRL/120	L26b	D	0.18	0.22	2	dPCR	-2.10	-2.03	c
NRL/625	L27	D	0.56	0.2	2	Real-time PCR	1.18	1.23	a
NRL/625	L28	D	0.47	0.14	2	Real-time PCR	0.40	0.55	a
NRL/625	L29	D	0.62	0.16	2	Real-time PCR	1.69	2.11	a
NRL/625	L30	D							
NRL/120	L31	D	0.48	0.43	2	Real-time PCR	0.49	0.26	c
NRL/120	L32	D	0.32	0.1	2	Real-time PCR	-0.89	-1.50	a
NRL/625	L33	D	0.79	0.24	2	Real-time PCR	3.16	2.84	a
NRL/625	L34a	D	0.42	0.16	2	Real-time PCR	-0.03	-0.04	a
NRL/625	L34b	D	0.41	0.1	2	dPCR	-0.11	-0.19	a
NRL/625	L35	D	0.36	0.06	2	Real-time PCR	-0.55	-1.13	b

LabType	LabCode	ID	x_i	±	k	Technique	z' score	ζ score	MU
NRL/625	L36	D	0.58	0.25	2	Real-time PCR	1.35	1.17	a
NRL/120	L37	D	0.51	0.087	2	Real-time PCR	0.75	1.34	b
NRL/120	L38a	D	< 0.9			Real-time PCR			
NRL/120	L38b	D	0.48	0.12	3.18	dPCR	0.49	0.93	b
NRL/625	L39	D	0.49	0.18	2	Real-time PCR	0.57	0.65	a
NRL/625	L40	D	0.21	0.03	2	Real-time PCR	-1.84	-4.27	b
NRL/625	L41	NT							
NRL/120	L42	D	1.83	0.61	3.18	dPCR	12.12	7.12	b
NRL/625	L43	NT							
NRL/625	L44	D	0.43	0.1	1.73	Real-time PCR	0.06	0.09	a
NRL/625	L45	NT							
OCL	L46	NT							
OCL	L47	D	0.66			Real-time PCR	2.04	4.97	NP
OCL	L48	NT							
OCL	L49	D	0.5			Real-time PCR	0.66	1.61	NP
OCL	L50	NT							
OCL	L51	ND							
OCL	L52	D	0.14	0.03	2	Real-time PCR	-2.44	-5.67	b
OCL	L53	D	0.45	0.3	2	Real-time PCR	0.23	0.17	c
OCL	L54	D	0.44	0.18	2	Real-time PCR	0.14	0.16	a
OCL	L55	NT							
OCL	L56	D	0.7	0.3	3	Real-time PCR	2.38	2.50	a
OCL	L57	D	0.4	0.094	2	Real-time PCR	-0.20	-0.35	a
OCL	L58	D	0.53	0.15	2.36	dPCR	0.92	1.34	a
OCL	L59	NT							
NRL/625	L60	D	0.38	0.09	2		-0.37	-0.66	a
NRL/625	L61	D	0.47	0.12	2	Real-time PCR	0.40	0.61	a
OCL	L62	D	0.41	0.12	2	Real-time PCR	-0.11	-0.17	a
OCL	L63	D	0.5	0.22	2	Real-time PCR	0.66	0.64	a
OCL	L64	NT							
OCL	L65	NT							
OCL	L66	ND							
NRL/625	L67	D	0.4	0.12	2	Real-time PCR	-0.20	-0.30	a
NRL/625	L68a	D	0.36	0.09	2	Real-time PCR	-0.55	-0.97	a
NRL/625	L68b	D	0.44	0.11	2	dPCR	0.14	0.23	a
OCL	L69	NT							
OCL	L70	NT							

*L22 reported “absent” in the reporting form, but provided a value



Upper right: kernel density distribution

A5547 soybean in T2

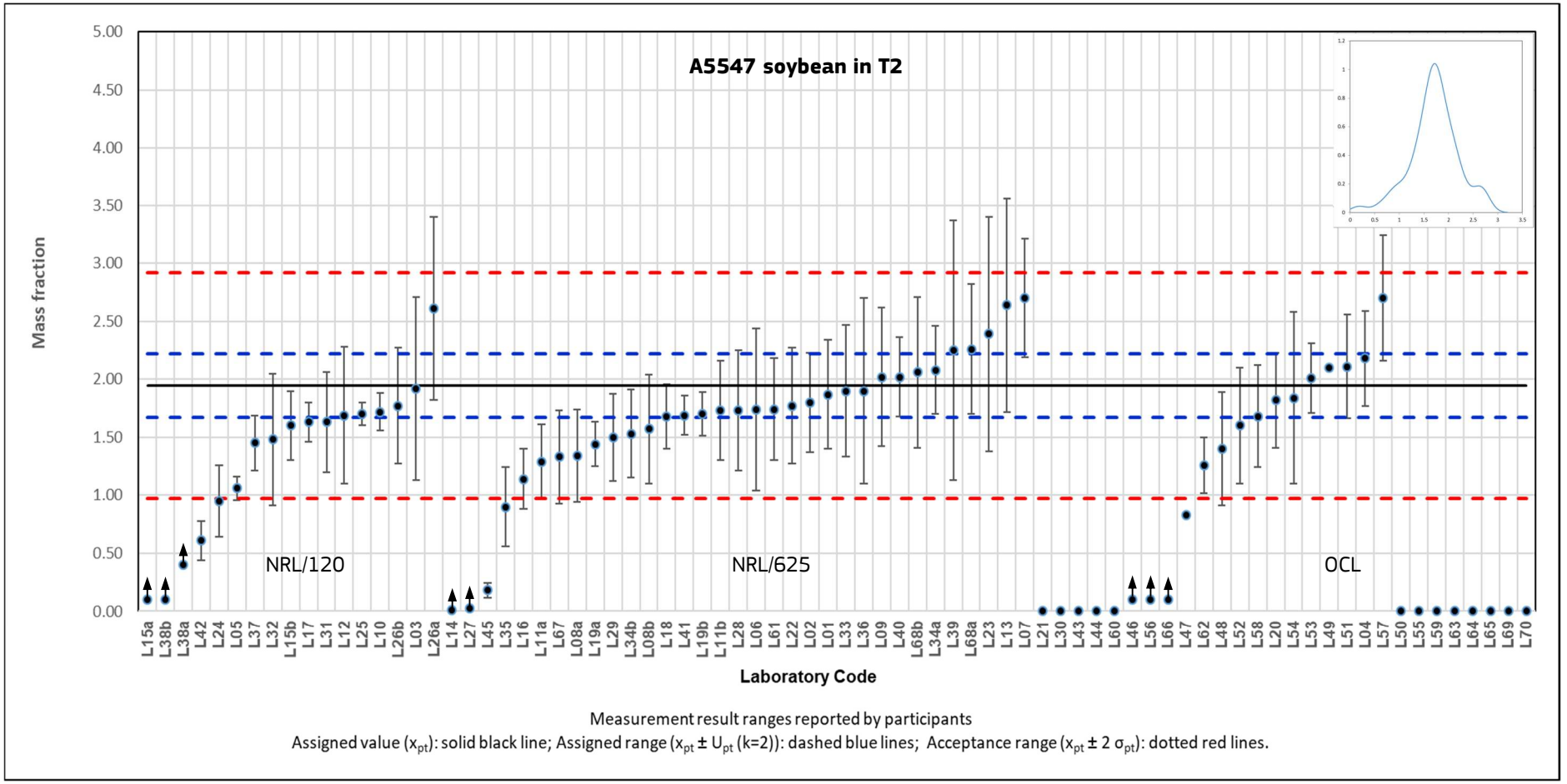
- ID = GM event identification (D = detected, ND = not detected, NT = not tested)
- The PT coordinator set the measurement uncertainty $u(x_i)$ to zero when no expanded uncertainty was reported
- The PT coordinator set k = 1.73 when no coverage factor (k) was reported
- Performance scores (z and ζ): satisfactory, questionable, unsatisfactory
- Measurement uncertainty (MU): a: $u_{rel}(x_{pt}) \leq u_{rel}(x_i) \leq \sigma_{pt,\%}$; b: $u_{rel}(x_i) < u_{rel}(x_{pt})$; c: $u_{rel}(x_i) > \sigma_{pt,\%}$; NP = not provided

Evaluation parameters: x_{pt} = 1.944 ; $u(x_{pt})$ = 0.136 ; σ_{pt} = 0.486 (all values in m/m %)

LabType	LabCode	ID	x_i	±	k	Technique	z score	ζ score	MU
NRL/625	L01	D	1.87	0.47	2	Real-time PCR	-0.15	-0.27	a
NRL/625	L02	D	1.8	0.43	2	Real-time PCR	-0.30	-0.57	a
NRL/120	L03	D	1.92	0.79	2.11	Real-time PCR	-0.05	-0.06	a
OCL	L04	D	2.18	0.41	2	Real-time PCR	0.49	0.96	a
NRL/120	L05	D	1.06	0.1	2	Real-time PCR	-1.82	-6.09	b
NRL/625	L06	D	1.74	0.7	2	Real-time PCR	-0.42	-0.54	a
NRL/625	L07	D	2.7	0.51	2	Real-time PCR	1.56	2.61	a
NRL/625	L08a	D	1.34	0.4	2	Real-time PCR	-1.24	-2.50	a
NRL/625	L08b	D	1.57	0.47	2	dPCR	-0.77	-1.38	a
NRL/625	L09	D	2.02	0.6	2	Real-time PCR	0.16	0.23	a
NRL/120	L10	D	1.72	0.16	2	dPCR	-0.46	-1.42	b
NRL/625	L11a	D	1.29	0.32	2	Real-time PCR	-1.35	-3.11	a
NRL/625	L11b	D	1.73	0.43	2	dPCR	-0.44	-0.84	a
NRL/120	L12	D	1.69	0.59	2	dPCR	-0.52	-0.78	a
NRL/625	L13	D	2.64	0.92	2	Real-time PCR	1.43	1.45	a
NRL/625	L14	D	> 0.01			Real-time PCR			
NRL/120	L15a	D	> 0.1			Real-time PCR			
NRL/120	L15b	D	1.6	0.3	2	dPCR	-0.71	-1.70	a
NRL/625	L16	D	1.14	0.26	2	Real-time PCR	-1.65	-4.27	a
NRL/120	L17	D	1.63	0.17	2	Real-time PCR	-0.65	-1.96	b
NRL/625	L18	D	1.68	0.28	2	Real-time PCR	-0.54	-1.35	a
NRL/625	L19a	D	1.44	0.19	2	Real-time PCR	-1.04	-3.03	b
NRL/625	L19b	D	1.7	0.19	3.18	dPCR	-0.50	-1.64	b
OCL	L20	D	1.82	0.41	2	dPCR	-0.26	-0.50	a
NRL/625	L21	NT				Real-time PCR			
NRL/625	L22	D*	1.77	0.5	2	Real-time PCR	-0.36	-0.61	a
NRL/625	L23	D	2.39	1.01	2	Real-time PCR	0.92	0.85	a
NRL/120	L24	D	0.95	0.31	2	Real-time PCR	-2.05	-4.82	a
NRL/120	L25	D	1.7	0.1	2	dPCR	-0.50	-1.68	b
NRL/120	L26a	D	2.61	0.79	2	Real-time PCR	1.37	1.59	a
NRL/120	L26b	D	1.77	0.5	2	dPCR	-0.36	-0.61	a
NRL/625	L27	D	> 0.025			Real-time PCR			
NRL/625	L28	D	1.73	0.52	2	Real-time PCR	-0.44	-0.73	a
NRL/625	L29	D	1.5	0.375	2	Real-time PCR	-0.91	-1.92	a
NRL/625	L30	D							
NRL/120	L31	D	1.63	0.43	2	Real-time PCR	-0.65	-1.23	a
NRL/120	L32	D*	1.48	0.57	2	Real-time PCR	-0.95	-1.47	a
NRL/625	L33	D*	1.9	0.57	2	Real-time PCR	-0.09	-0.14	a
NRL/625	L34a	D	2.08	0.38	2	Real-time PCR	0.28	0.58	a
NRL/625	L34b	D	1.53	0.38	2	dPCR	-0.85	-1.77	a
NRL/625	L35	D	0.9	0.34	2	Real-time PCR	-2.15	-4.79	a

LabType	LabCode	ID	x_i	±	k	Technique	z score	ζ score	MU
NRL/625	L36	D	1.9	0.8	2	Real-time PCR	-0.09	-0.10	a
NRL/120	L37	D	1.45	0.24	2	Real-time PCR	-1.02	-2.72	a
NRL/120	L38a	D	> 0.4			Real-time PCR			
NRL/120	L38b	D	> 0.1			dPCR			
NRL/625	L39	D	2.25	1.12	2	Real-time PCR	0.63	0.53	a
NRL/625	L40	D	2.02	0.34	2	Real-time PCR	0.16	0.35	a
NRL/625	L41	D	1.69	0.17	2	Real-time PCR	-0.52	-1.58	b
NRL/120	L42	D	0.61	0.17	3.18	dPCR	-2.74	-9.11	a
NRL/625	L43	NT							
NRL/625	L44	D							
NRL/625	L45	D	0.18	0.063	2	Real-time PCR	-3.63	-12.61	a
OCL	L46	D	> 0.1			Real-time PCR			
OCL	L47	D	0.83			Real-time PCR	-2.29	-8.18	NP
OCL	L48	D	1.4	0.49	2	Real-time PCR	-1.12	-1.94	a
OCL	L49	D	2.1			Real-time PCR	0.32	1.14	NP
OCL	L50	D				Real-time PCR			
OCL	L51	D	2.11	0.45	2	Real-time PCR	0.34	0.63	a
OCL	L52	D	1.6	0.5	2	Real-time PCR	-0.71	-1.21	a
OCL	L53	D	2.01	0.3	2	Real-time PCR	0.14	0.33	a
OCL	L54	D	1.84	0.74	2	Real-time PCR	-0.21	-0.26	a
OCL	L55	NT							
OCL	L56	D	> 0.1			Real-time PCR			
OCL	L57	D	2.7	0.54	2	Real-time PCR	1.56	2.50	a
OCL	L58	D	1.68	0.44	2.09	dPCR	-0.54	-1.05	a
OCL	L59	NT							
NRL/625	L60	NT							
NRL/625	L61	D	1.74	0.44	2	Real-time PCR	-0.42	-0.79	a
OCL	L62	D	1.26	0.24	1.73	Real-time PCR	-1.41	-3.52	a
OCL	L63	NT							
OCL	L64	NT							
OCL	L65	NT							
OCL	L66	D	> 0.1			Real-time PCR			
NRL/625	L67	D	1.33	0.4	2	Real-time PCR	-1.26	-2.54	a
NRL/625	L68a	D	2.26	0.56	2	Real-time PCR	0.65	1.01	a
NRL/625	L68b	D	2.06	0.65	2	dPCR	0.24	0.33	a
OCL	L69	NT							
OCL	L70	NT							

*L22, L32 and L33 reported “absent” in the reporting form, but provided a value



Upper right: kernel density distribution

Annex 6. Results of the questionnaire

The answers to the questionnaire are presented in the tables below. Note that in some cases only the most informative answers to open questions are shown or a summary of the answers is provided. As some laboratories reported both qPCR and dPCR results, the numbers shown refer to the number of answers, not the number of laboratories.

Please select which test items were analysed by your laboratory (Note: if you select "yes" to one of the test items, several further questions will pop up; if you select "no", a further question will pop up for the selected test item asking to indicate the reason for your answer)

Answer	T1	T2
Yes	68	69
No	2*	1*
No Answer	0	0

*L50 did not analyse T1, L15(b) did not analyse T1 by dPCR, L60 did not analyse T2

Please explain why T1 and/or T2 were not analysed

Justification	T1	T2
a) The sample matrix is out of the scope of our laboratory	1	1
b) The methods are not validated in our laboratory	1	0
c) We could not obtain sufficient good quality DNA suitable for further analysis	0	0
d) Appropriate Certified Reference Material was not available	0	0
e) Primers, probes, or other reagents were not available (in time)	0	0
f) We tried but our analysis failed	0	0
g) Other practical constraints (instrument broken, no personnel, etc.)	0	0
h) Other reason	0	0
No Answer	68	69

Are the methods used within the scope of accreditation of your laboratory under ISO/IEC 17025?

Method	Test Item	Yes	No	Partially	Not applicable
DNA extraction method	T1	63	4	0	1
	T2	65	3	0	1
Qualitative GMO screening method(s)	T1	57	5	5	1
	T2	58	3	7	1
Qualitative event-specific identification method(s)	T1	45	11	12	1
	T2	48	7	14	1
Quantitative event-specific GMO method(s)	T1	36	19	7	4
	T2	42	16	8	4

Further explanation on the work not done under accreditation

Not yet flexible scope, several targets (QL/QN) not under accreditation
Previous methods in pipeline for accreditation, recent methods in verification.
The inclusion of the MIR604 method /quantitative) in the scope of accreditation will be requested at the next annual audit
Qualitative event specific identification method not validated used for analysis of T1: Mir162 and T2: 5547 and DAS81419; Quantitative event specific GMO method not validated used for analysis of T2: 5547
dPCR used only for quantification. Not accredited
Following events are not accredited: GA21, MON87429, CV127, and MON87769
Several dPCR methods and technology itself are in scope of accreditation, but not the events tested;
Both QN method were modified (use of different qPCR mix) and validated as such, but are not yet formally included in the quality system
Accreditation of screening elements (Cry1Ab/Ac, pNOS-nptII and pNOS) is planned for 2023-2024
Digital PCR as a new method to quantify GMO is not yet accredited.under ISO/IEC 17025:2017 in our laboratory. The accreditation is in process and will be completed this year.
Methods are not all included in our scope of accreditation.
Still waiting of the finishing of the accreditation-procedure for the ddPCR-method (more than one year!!)
Quantification using ddPCR will be under accreditation after the next DakkS Audit.
Popcorn is out of our matrix accreditation (food - not feed as we are able to do under ISO 17025)

Since we do not have all methods accredited, we do not have all primers and probes for all strains.
The detection/identification of soybean event A5547 is validated for feed in our laboratory but not the quantification of this event
soja A5547 jest w trakcie walidacji
The sample matrices are out of the scope of our laboratory
T2 - method available, but not fully validated because no certified reference material was available in the laboratory
Only MON863 quantitative event-specific method is under ISO/IEC 17025
Our lab is accredited under the flexible scope. Events MIR604 and A5547 were for the first time used, therefore they were not yet reported to the Accreditation body.
For T2, only GTS 40-3-2 is within the scope of accreditation.
Accredited methods: P35, Tnos, ADH, Lecitin; Non accredited methods: T9, DAS40278/9, Pat, Bt11

What was the approximate sample intake used for DNA extraction (in mg powder)?

	500 mg	**400 mg**	**300 mg**	**200 mg**	**150 mg**	**100 mg**	**<100 mg**
T1	14	0	5	49	0	0	1
T2	16	0	4	47	0	3	1

Select the DNA extraction method and any additional purification method(s) used for T1 and T2

DNA extraction method	**T1**	**T2**
CTAB method with 1% CTAB in lysis buffer	5	5
CTAB method with 2% CTAB in lysis buffer	21	21
Maxwell RSC PureFood GMO and authentication kit	5	6
Maxwell RSC/LEV Plant DNA kit	1	1
NucleoSpin Food	18	17
NucleoSpin Plant	1	2
NucleoMag DNA Food	1	1
NucleoMag DNA Plant	0	0
GeneSpin	6	6
Wizard genomic DNA purification kit	0	0
Qiagen DNeasy Plant	1	1
Qiagen DNeasy Mericon Food	3	3
Qiagen Blood and Tissue kit	1	1
Biotecon Foodproof	2	2
SDS	2	1
Speedtools Food DNA extraction kit (Biotools)	2	2
Generon Ion Force	0	0
Eurofins DNAExtractor cleaning column	4	4
Promega Wizard DNA clean-up resin	2	2
Qiagen QIAQuick	3	3
Qiagen Genomic-Tip 20/G	0	0
NucleoSpin gDNA clean-up	0	0
Zymo OneStep PCR inhibitor Removal kit	1	1
Qiaex II purification kit	1	1

Please indicate below any important details or modifications to the DNA extraction method(s) used.

RNase treatment
CTAB precipitation method
combined Maxwell-CTAB method
Pre-step with Lysing Matrix A. Qiagen Blood and Tissue kit: The solutions were added 4 times the volume indicated in the kit.
CTAB lysis with magnetic bead clean up (NucleoMag Plant)
The sample lysate has been applied to the column several times to increase the yield.
For T2: Zymo Quick-DNA Kit was used a second extraction method
Increased amount of sample and volume of lysis buffer

for T1 CTAB concentration is 1,4%
T2 CTAB method with 2% CTAB in lysis buffer plus Qiagen QIAQuick purification
longer incubation time, bigger volume of CF buffer, for T2 sample we use NucleSpin Filters ref 740606
We used the NucleoMag Food Kit for T1 and T2
A CTAB lysis was performed prior to the DNA extraction with MN NucleoSpin Food Kit
Mobispin clean up
For T2 the volume of lysis buffer and proteinase K were increased.
r-biopharm Surefood PREP Basic

Did you verify absence of PCR inhibition in the extracted DNA?

Answer	**T1**	**T2**
No	10	11
We performed a PCR inhibition test on a reference gene target prior to the analysis (using 2 or more dilutions)	29	29
We performed a PCR inhibition test on a GM gene target prior to the qPCR analysis (using 2 or more dilutions)	4	4
We analysed two or more dilutions of the DNA and compared the results	26	26
An internal positive control was added to the unknown samples	11	11
Other	3	3

Provide further clarification on the approach used for DNA quality analysis and the outcome

Always have a critical look on the amplification curves
PicoGreen measurement
The extracted DNA was diluted to 20ng/uL and a further 1:4 dilution was prepared. Both dilutions were amplified using a suitable reference gene qPCR assay (T1: hmg, T2: lec). The delta Cq-value was assessed for PCR inhibition (expected delta Cq +/-0.5).
DNAs were checked by spectrophotometry (Nanodrop). Maize samples usually do not exhibit inhibition. Therefore, this was not tested. DNAs from T2 (analysed in triplicate) were used only for screening and identification. The two events with higher probability to be present were not tested because the CRMs did not arrive in due time (A2704-12 and A5547). Due to this limitation, we did not spend more resources testing for inhibition.
we perform inhibition test on reference gene target during the screening and inhibition test of the reference gene target and event-specific target during the quantification. Three dilutions are run.
the samples and the 1:4 dilutions were tested in the same PCR run and the obtained results were compared
Qubit dsDNA BR kit was used to measure DNA quantity and quality, inhibition tests run twise with different template µl to estimate optimal template concentration for quantifications.
DNA quality check: ratio of absorbance and in the course of the PCR inhibition controls and at least in two dif ferent DNA concentrations (if possible with 40 µg/µL and diluted 1:4). No inhibition could be observed for either sample.
Measurements with spectrophotometer Nanodrop ND-1000
DNA nanodrop measurement, A260/A280 result
The extracted DNA was diluted to 40 ng/µl and a further 1:4 dilution was prepared. Both dilutions were analyzed and qPCR results were compared.
We used: negative and positive extraction and PCR controls, inhibition absence was tested with similar items in methods validation
EURL approach followed i.e. 1:4 - 1:16 dilutions

Do you consider the DNA extracted from T1 and T2 as suitable for quantitative PCR analyses?

	T1	**Ratio**	**T2**	**Ratio**
Yes	66	94.29%	64	91.43%
No	0	0%	0	0%
Not sure	0	0%	2	2.86%
Not applicable	3	4.29%	3	4.29%
No Answer	1	1.43%	1	1.43%

If screening methods were used (excluding species and event-specific methods), please indicate the results (presence or absence).

Screening target	T1: present	T1: absent	T2: present	T2: absent	No Answer
P35S	2	60	62	0	5
tNOS	62	1	3	56	5
PAT	1	41	47	0	21
BAR	0	34	0	32	35
CP4-EPSPS	0	5	0	5	64
Ctp-CP4-EPSPS	0	0	0	1	69
Ctp2-CP4-EPSPS	0	32	0	35	32
Cry1Ab/Ac	0	17	1	19	50
Cry1Ab	0	0	0	0	70
pFMV	3	27	3	26	38
pNOS	0	3	0	3	67
t35S	0	2	1	1	68
nptII	0	6	0	7	63
p35S-pat	0	7	11	0	58
p35S-nptII	0	1	0	0	69
pCsVMV-pat	0	2	0	3	67
tE9	0	10	0	16	54
Agrobacterium border seq.	4	4	0	5	63
CaMV	0	3	0	2	67
Other	0	2	0	1	68

Further details on other screening targets

T1:analysis for MON95379 with pFMV-Screening, other screenings: event-specific multiplex real-time PCR for DAS40278/VCO-01981-5/MON87419 (events without p35S and/or tNOS)
T1: Agroborder I = present, Agroborder II = absent;
detection of pNOS-nptII - 2x absent
Together with the screening, the following events were tested with the event-specific method because they were not covered by our screening approach: T1:1981, 40278, LY038, 95379; T2: 87708, 87751, 305423, CV127, 87769
We performed event specific screening
34S FMV not detected in both samples
T-orf23
Event-specific methods (real-time PCR multiplexing)
To identify the GM event in T1, a screening was done using GM maize event-specific tetraplex qPCR assays. To identify the GM soy in T2, a screening was done using GM soy event-specific tetraplex qPCR assays.

If your laboratory did not perform tests for all relevant GM events (e.g. only screening and/or some event-specific tests), how do you report the results to your customer (Competent Authority or other)? Select all applicable answers.

	Answers	Ratio
We report the results of the screening tests performed	55	78.57%
We report the results of the event-specific tests performed	52	74.29%
We indicate the GM events that were not tested	3	4.29%
We send the samples to another laboratory for further testing	13	18.57%
No Answer	12	17.14%

Further details on the way of reporting

Since March this year, we also report the GM events that were not tested but had some probability to be present. The results of the screening and the identification are analysed in detail and communicated to the client. If the lab hasn't the corresponding CRM, primers and probes, or if the method was never implemented and verified, the client is informed of the time needed to implement and verify the method before being able to give a final result. It will be up to client to bring the sample to other laboratory.
We report the results in the screening step and results of GM event PCRs. As technical information we report GM events that cannot be present in the sample as a result of the screening.

Since we do not have all methods accredited, we do not have all primers and probes for all GM events.
Screening elements are only reported if they cannot be assigned to an GM event or a donor organism
For this PT applied screening and event-specific tests covered all registered GM events and pending/withdrawn (covered by regulation 619/2011) GM events. We send samples to another laboratory for further testing but when all registered and pending/withdrawn (regulation 619/2011) cannot be covered by validated testing we would indicate GM events that were not tested.
We test with a set of transgenic screening markers+events not covered by screening markers that cover all authorised events, all events under 619/2011, known non-authorised events. In identification all potentially present events are tested, so we test everything that is requested by our CA
All relevant GM events are in our scope.
The relevant scope of the analyses is discussed with our customers
We always perform full screening and identification and quantification analysis for all possible events.
Online reporting to food control authorities via web interface
In our report we indicate the screening and the event-specific test performed and, furthermore, we send the samples to the National Reference Laboratory. In the case of this specific test, in view of our results, we can deduce that the GM that each sample may have are: T1: 3272 Maize, 5307 Maize, GA21 Maize, MIR162 Maize or/and MIR604 Maize; T2: A2704-12 or/and A5547-127 Soybean.
If we have relevant information for our CA, we contact them through mail and inform there. Normally we have no missing methods mentioned on the certificates.

Provide the full code of the CRM(s) used for quantification (for calibration or as QC material).

Test Item	CRM Producer	CRM Code	Answers
T1	JRC	ERM-BF423(d)	53
T2	AOCS	0707-C(2 to 8)	51

Other answers: Bayer CropScience 4-07-b00426/2007 (L15b dPCR for T2); Gold Standard Technologies EUS5211512801 (L53 for T2) or 5125220601 (L18 for T2 as QC material)

Specify the taxon-specific reference target(s) used for quantification, if applicable.

Test item	Reference target	Answers
T1 – MIR604	Maize *hmg*	37
	Maize *Adh1* – 134/136 bp	21
	Maize *Adh1* – 70 bp	2
	Maize Invertase	0
	Other	0
T2 – A5547	Soybean *Le1* (70 bp) - QT-TAX-GM-004	0
	Soybean *Le1* (74 bp) - QT-TAX-GM-002	31
	Soybean *Le1* (102 bp) - QT-TAX-GM-003	4
	Soybean *Le1* (81 bp) - QT-TAX-GM-001	4
	Soybean *Le1* (70 bp) - QT-TAX-GM-004	0
	Soybean *Le1* (102 bp) - QT-TAX-GM-020	9
	Soybean *Le1* (105 bp) - QT-TAX-GM-009	2
	Soybean *Le1* (118 bp) - QT-TAX-GM-007	5
	Other*	4

*QT-TAX-GM-005; SOJA LEKTIN (L36), Sltm1, Sltm2, Sltmp 80 bp, Va M, Pijnenburrg H, Gendre F, Brignon P (1999) J Agric Food Chem 47:5261-5266 (L16)

When using digital PCR, which general system did you use?

Digital PCR system	Answers
Droplet dPCR, total	12
BioRad QX100	1
BioRad QX200	11
Chamber dPCR, total	2
Qiagen QIAcuity	2

Did you modify the final concentration of primers and probes when using dPCR compared to the validated qPCR method? If so, to which concentration and why?

Answer	No of answers	Clarification
No	7	the same concentration of real time PCR (0,6 pmol/µl, 0,3 pmol/µl, 0,2 pmol/µl)
Yes	1 (QIAcuity)	the assay used is a multiplex assay and the primer and probes are 600 nM - 600 nM and 250 nM
	1	slightly higher concentration of probe and slightly lower concentration of primers - better performance, e.g. very low rain
	1	yes: primers and probes according to the manufacturers recommendations (primers: 900 µM; probes. 250 µM)
	1	yes, we used concentration for the primer of 500nM and for the probe of 100 nM. That is our standard ddPCR protocol.
	1	no modification of dPCR compared to qPCR; for both: primer conc. 0.4 uM, probe conc. 0.2 uM (but maybe modification compared to EURL qPCR reference methods)
	1	For improved discrimination of the droplet populations, the primers and probes were used at a final concentration of 450 nM (for primers) and 125 nM (for probes), respectively.

Provide details of any conversion factor used to convert your results for T1 and T2 from GM copy number ratio to GM mass fraction (e.g. when using dPCR).

7 answers: Conversion factors (CFcrm) listed in the document "Conversion factors (CF) for certified references materials (CRM) (version 9 - 16/02/2023)", i.e. 0.448 for MIR604 and 0.99 for A5547
2 answers: T1: Conversion factor=0.45; T2: Conversion factor=1.01
CF for T1: 0,49; CF for T2: 1
Our result GM copy number x 2 = our result GM mass fraction (for T1)
T1: 0.460; T2: 0.969
T1 : 0,448 - T2 : 1
MIR604: 0.45; A5547-127: 0.98
T1 (MIR604/hmg): CF=0.400 / T2 (A5547/lec): CF=0.955

If you are using dPCR for GMO measurements in your lab, what are the main reasons for choosing dPCR instead of qPCR?

	Answers	Ratio
Lower sample intake	0	0%
Less affected by PCR inhibitors	12	17.14%
No CRM stock needed	7	10%
Time of analysis shorter	2	2.86%
Cost of analysis less	0	0%
Other	3	4.29%
No Answer	57	81.43%

Feel free to mention other reasons for using dPCR

Better trueness and precision
We would like validate and accreditate the ddPCR in addition to the real time PCR. The PT results are useful to the accreditation body.
No quantified CRM available
No standard curve necessary when quantifying by dPCR.
Lower measurement uncertainty compared to real-time PCR
No standard curve quantification needed any more (no qPCR repetitions due to underperforming standard curves).

Based on your measurement results do you consider the sample compliant with the EU GMO legislation, considering that the sample was derived from a product not declared as containing GM material?

See Tables 6 and 7 of this report.

Please justify the answers provided above (only a few selected answers are shown).

As we did not receive the CRMs for A2704-12 and A5547-127 in due time, we could not identity and quantify. Both events are authorized, but the stack is unauthorized. The primary reason why we could not conclude was the incapacity to test and determine if one or both were present and in what amount; if we would be able to identity and quantify, then if both would be present, we wouldn't be able to conclude because we would not know if it was a stack or not.
For T1, MIR604 is an authorized maize and it is present below 0.9 m/m%, Since the rest of maize events in situation of authorized, withdrawn or pending have not been detected or concluded that they are absent, it can be deduced that the product complies with the regulations and labelling is not necessary. For T2, A-5547 is a recently authorized soybean and is over 0.9% (including the Uncertainty). Only with this analysis it can be established that it is necessary labelling of the product and therefore does not comply with the EU legislation.
Not relevant for Turkey
During the detection and identification of GMO in sample T1 we confirmed presence of authorised maize event MIR604. The measured value is 0.68, after the subtraction uncertainty from our result, value of result is below 0.9% and after addition uncertainty - the value is above 0.9, measured value is 0.68, and the lowest value is 0.32, it means, that the sample is compliant with the EU GMO legislation and labelling is not required. During the detection and identification of GMO in sample T2 we confirmed presence of authorised soybean event A5547. From our measurement result and after the subtraction uncertainty from our result, value of result is above 0.9% - it means, that the sample is not compliant with the EU GMO legislation and labelling is required.
T1: CNL; sample contains an authorized GM event below labelling threshold of 0.9%, therefore it is compliant and no labelling is required. In the present case, the content of GMO incl. MU is above the labelling threshold, the manufacturer or the production would be checked. This also includes a plausibility check to verify the adventitious and technically unavoidable presence. T2: NCL (X+/- U > 0.9%)
Both events are allowed on EU markets as food and feed (not for cultivation). T1 sample compliant to regulation 1829/2003, as GMO % is below labelling limit, even with our expanded MU [0,48 m/m% + (0,48 m/m% *0,43) = 0,6864 m/m% --> 0,7 m/m%]. T2 sample is technically just over labelling limit (even when considering our expanded MU [1,63 m/m% - (1,63 m/m% *0,43) = 0,9291m/m% = 0,93 m/m%. However considering the decimal accuracy in the regulation, the result of T2 sample rounds down to 0,9 m/m%, which would make it compliant. I would leave it to our national monitoring officials to make the final desition on the sample compliancy.
We have detected t-NOS in T1 sample, and P35S in T2 sample. That means that there is genetically material modified. We only have analysed MON810 in T1 sample and soybean 40-3-2 in T2 sample, both results are not detected. We need more information to say if the samples are compliant.
Our laboratory is not authorized to conclude. Only our ministry can do it.
T1: amount below the limit; T2 above the limit using AOCS-CRM, uncertainty due to different extraction of CRM and sample T2, due to different ploidy between leaf tissue and seeds and due to unknown zygosity of sample T2 have to be emphasized!

Additional comments and suggestions

Good idea to extend the deadline one more week as still some analysis in process.
The analysis of T2 was performed by Chemical and Veterinary Analytical Institute Rhein-Ruhr-Wupper (cooperating institute).
Compliance assessment based only on events, that are authorized in EU; this task should be clarified beforehand
In T1 we identified also MON863 in very low concentration.
The values zero mean of course that no result was generated above the detection limit of 0.03 percent
Nice round, thanks!
In sample T1 we have found traces when screening for P35S (200 ng/PCR Ct values for the first Extract 42.65 / 35.21 and for the second Extract 42.82 / 41.23). The Ct values are under the limit of Detection.
CRM from AOCS was provided as DNA.solution: --> no extraction possible. T2 was provided as flour, no testing for zygosity was possible.
Only make the droplet PCR part visible if a box ticks that you use it.

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The portal data.europa.eu provides access to open datasets from the EU institutions, bodies and agencies. These can be downloaded and reused for free, for both commercial and non-commercial purposes. The portal also provides access to a wealth of datasets from European countries.

Science for policy

The Joint Research Centre (JRC) provides independent, evidence-based knowledge and science, supporting EU policies to positively impact society

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