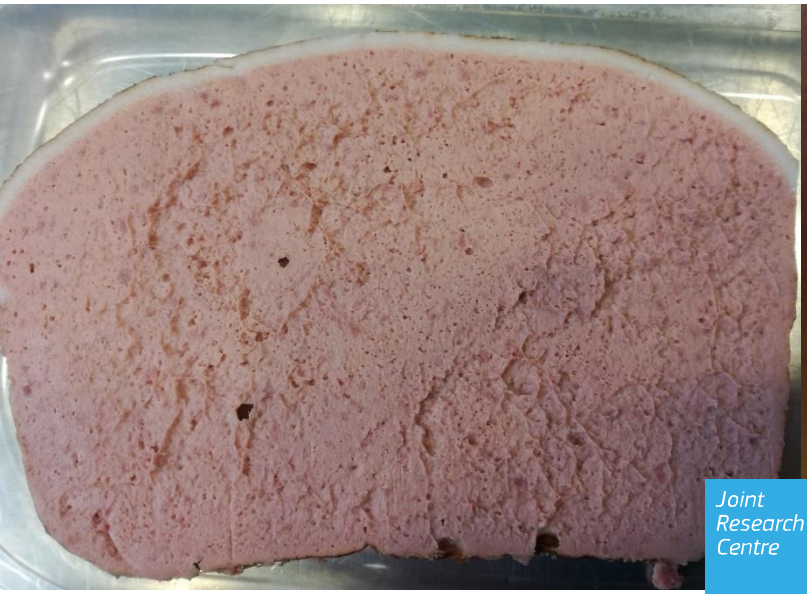
European Commission

JRC VALIDATED METHODS, REFERENCE METHODS AND MEASUREMENTS REPORT

Determination of GM Soybean MON89788 in Meat Pâté (T1) and GM Maize T25 in Maize Flour (T2)

EURL GMFF Proficiency Testing Report GMFF-21/02

Broothaerts, W., Beaz Hidalgo, R., Buttinger, G., Corbisier, P., Cubria Radio, M., Dehouck, P., Emteborg, H., Maretti, M. and Robouch, P.



Joint Research Centre

EUR 31103 EN

Contact information
European Union Reference Laboratory for GM Food and Feed
European Commission, Joint Research Centre
Retieseweg 111, B-2440 Geel - Belgium
Email: JRC-EURL-GMFF-CT@ec.europa.eu

EU Science Hub
https://ec.europa.eu/jrc

JRC128221

EUR 31103 EN

PDF ISBN 978-92-76-53107-4 ISSN 1831-9424 doi:10.2760/68798

Luxembourg: Publications Office of the European Union, 2022

How to cite this report: Broothaerts, W., Beaz Hidalgo, R., Buttinger, G., Corbisier, P., Cubria Radio, M., Dehouck, P., Emteborg, H., Maretti, M. and Robouch, P. *Determination of GM Soybean MON89788 in Meat Pâté (T1) and GM Maize T25 in Maize Flour (T2). EURL GMFF Proficiency Testing Report GMFF-21/02,* EUR 31103 EN, Publications Office of the European Union, Luxembourg, 2022, ISBN 978-92-76-53107-4, doi:10.2760/68798, JRC128221.

Determination of GM Soybean MON89788 in Meat Pâté (T1) and GM Maize T25 in Maize Flour (T2)

EURL GMFF Proficiency Testing Report
GMFF-21/02

Broothaerts, W., Beaz Hidalgo, R., Buttinger, G., Corbisier, P., Cubria Radio, M., Dehouck, P., Emteborg, H., Maretti, M. and Robouch, P.

268-PT Accredited by the
Belgian Accreditation Body (BELAC)

Contents

Executive summary

The European Union Reference Laboratory for Genetically Modified Food and Feed (EURL GMFF) organised a proficiency testing (PT) round (GMFF-21/02) for the determination of GMOs in food and feed materials to support Regulation (EU) 2017/625 on official controls [1]. This PT, managed in line with ISO 17043:2010 [2], was open to National Reference Laboratories (NRLs) and EU official control laboratories (OCLs). Few additional officially appointed laboratories from outside the EU were accepted as well.

Two proficiency test items were distributed to participants to assess the efficacy of GMO analysis in meat pâté contaminated with soybean (T1) and in maize flour (T2).

T1 consisted of a locally purchased meat pâté spiked with MON89788 soybean powder (MON-89788-1). As it was the first time that a meat-based material was used in the PT scheme, the analysis of T1 was proposed as a feasibility study. The determination of the assigned value required optimisation of both the DNA extraction procedure and the PCR assay, i.e. using hot-start real-time PCR or digital PCR, to decrease the effect of PCR inhibition and/or interference from the meat matrix.

T2 was composed of ground maize seed spiked with GM maize event T25 (ACS-ZMØØ3-2). The EURL GMFF evaluated the homogeneity and stability of the test items and derived the assigned values from in-house measurements.

Sixty one laboratories participated to the PT round, including 50 NRLs from 24 EU Member States, 8 EU OCLs and 3 official testing laboratories from EU-neighbouring countries.

The correct identification of the GM event in the two test items was evaluated. The quantitative results reported for the MON89788 event in T1 were compared to the assigned value using the % Difference (*D%*). The results reported for the T25 event in T2 were evaluated using *z* and zeta (ζ) scores in accordance with ISO 13528:2015 [3]. The relative standard deviation for proficiency assessment (σ_{pt}) was set to 25 %, based on the experience acquired from previous PT rounds. Also the compliance assessment of the samples in line with the reported quantitative results was evaluated.

All but one of the 51 laboratories who tested T1 identified the MON89788 soybean event, while all of the 58 laboratories who tested T2 identified the T25 maize event. The majority of the quantitative results reported for T1 were slightly below the assigned value, but did not deviate more than 50 % from it. For T2, 33 results were scored as satisfactory, 2 as questionable and 15 (30 %) as unsatisfactory. Thirteen of the unsatisfactory results were overestimated by a factor of 2 to 5. Further investigations by the EURL GMFF and some of the participants have excluded some sources of these unsatisfactory results, but further experiments are still ongoing and will be reported later as an addendum to the report.

More than 84 % of the participants correctly evaluated the compliance status of the two test items based on their reported measurement results.
This PT once more identified particular analytical issues that are not necessarily revealed by control laboratories during their routine analysis of samples.

List of abbreviations and symbols

bp	Base pairs
(d)dPCR	(Droplet) digital Polymerase Chain Reaction
DG SANTE	Directorate General for Health and Food Safety
EC	European Commission
EU	European Union
EURL	European Union Reference Laboratory
GMFF	Genetically Modified Food and Feed
GUM	Guide for the Expression of Uncertainty in Measurement
ISO	International Organization for Standardization
JRC	Joint Research Centre
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
k	Coverage factor
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*"
$U(x_{pt})$	Expanded uncertainty of the assigned value
x_i	Mean value reported by participant "*i*"
x_{pt}	Assigned value
$D\%$	Percentage difference
z	*z* score
ζ	zeta score

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 MON89788 soybean in meat pâté and T25 maize in maize 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 2021, 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 samples were prepared and dispatched to participants for analysis. A meat pâté (food test item T1) was selected since it may inadvertently contain traces of soybean. Similar products triggered already an emergency recall in the US in 2019 (https://www.foodsafetynews.com/2019/03/pate-recall-for-milk-and-soy-allergens-not-declared-on-label) because of the undeclared presence of soy allergens. The T1 matrix used here was spiked with GM soybean. Since such a new type of matrix was investigated for the first time in the EURL GMFF PT scheme, its analysis was presented as a feasibility study for which no performance scoring was foreseen. The second sample (feed test item T2) consisted of maize flour spiked with a GM maize event, which may be used as ingredient in various feed products.

This report presents the outcome of the PT.

2 Scope

The present PT round 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 a number of 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-21/02**".

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 participants in this PT received a unique laboratory code used throughout this report. 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. However, the laboratory codes of NRLs appointed in line with Regulation (EU) 2017/625 [1] may be disclosed to DG SANTE 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

The organisation of the GMFF-21/02 exercise was announced by invitation letters to NRLs and some accepted OCLs on August 27, 2021 (Annex 1). The registration deadline was set to September 12, 2021. Samples were sent to participants on September 28, 2021. The reporting deadline was set to November 12, 2021.

3.4 Distribution

Each participant received:

- One sachet with a bottle of test item T1, containing approx. 10 g of frozen meat pâté;
- One bottle of test item T2, containing approx. 5 g of dry powder;
- One general "Test item accompanying letter" (Annex 2).

Samples were dispatched frozen in the presence of dry ice.

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.

The test items were described as "*two ground test materials, derived from products that are not declared as containing GM material*". The testing laboratories were requested to screen for the presence of GMOs and assess the compliance of the samples with the applicable GMO legislation.

Participants were asked to check whether the bottles were damaged after transport and to store the test items in a dark and cool place at approximately -20 °C (T1) or 4 °C (T2). Additional information was provided to avoid DNA degradation in the wet matrix of T1, i.e. to keep the material at low temperature during thawing (e.g. on ice or in the fridge) and after sampling, until addition of the lysis buffer of the extraction method used. The unused portion of the sample should be stored in the fridge up to 5 days or returned to -20 °C for future needs (repeated freezing/thawing did not seem to affect the GM content measured).

Participants were requested to perform the following analyses:

T1: Meat pâté:	Verify the presence of GM soybean in this sample; Quantify the (single) GM event identified and assess compliance of the sample.
T2: Maize flour:	Verify the presence of the following GM maize events: 3272, 5307 and T25; Quantify the (single) GM event identified and assess compliance of the sample.

Participants were requested to report their calculated mean (x_i) and the associated expanded measurement uncertainty ($U(x_i)$) together with the coverage factor (k) and the analytical technique used for analysis.

Quantitative results had to be reported in mass/mass %. Since the homogeneity study was performed with 100 or 200 mg sample intakes for T1 and T2, respectively, the recommended minimum sample intake was set to these amounts.

Participants were informed that the procedure used for the analysis should resemble as closely as possible their routine procedures for these types of matrices. However, for T1, the participants were free to either apply their routine method(s) for DNA extraction or to perform further investigations in order to find a suitable method for this meat pâté matrix. Details of the measurement procedure used were to be reported in the questionnaire.

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

Participants were asked to fill in an online questionnaire through EU Survey, accessible with a provided password. The questionnaire was designed to collect additional information related to the measurements and the laboratories, including on the identification (qualitative analysis) of the GM event in both test items.

4 Test item

4.1 Preparation

Test item T1 consisted of a fresh cream meat pâté, purchased at a local supermarket, that was spiked with non-GM soybean seeds and GM soybean event MON89788 (12 % (m/m) soybean per total (i.e. dry soybean + wet pâté) mass, nominal MON89788 content: 1.5 % (m/m) – see Table 1 for details). The ground GM and non-GM soybean powders were mixed first, then the fresh meat pâté (without the fat layer) was added, and the materials were further mixed at room temperature to obtain a homogeneous mixture. The mixture was then manually filled into 50 mL glass vials (10 g per vial). Each vial, identified with the PT identifier and a unique vial number, was placed into an aluminium sachet and stored at -20 °C. The final T1 mixture had a water content of 6.0 ± 0.9 g/100 g (k=2, n=3) and an average particle diameter of 72.2 ± 1.6 µm (k=2, n=3).

The amount and the quality of the DNA extracted from the T1 material were verified by UV spectrometry, fluorometry and gel electrophoresis. The extracted DNA had a high concentration (100-300 ng/µL based on Picogreen measurements) and was partially degraded (Figure 1). The DNA extraction procedure was modified in order to reduce the amount of PCR inhibiting or interfering compounds in the extracts (see Section 4.2).

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

Characteristic	Meat pâté	Non-GM soybean	MON89788 soybean
Type of base material	Fresh product	Whole soybean	Powder
Origin	Aoste Cremepâté (Pâté Crème), purchased at Colruyt, Mol, Belgium	Pit & Pit (BE) Bio–Organic Soybeans	AOCS 0906-B2
Grinding equipment	/	Cryo-grinding vibrating mill	/
Mixing equipment	Stephan UM12 mixer		
Water content in g/100 g, mean ± U (k=2, n=3)	/	2.4 ± 0.2	/
Particle diameter in µm, mean ± U[1] (k=2, n=3)	/	108.3 ± 13.0	76.2 ± 20.6
Mass used to prepare T1 (g) – STEP 1		196.65	2.99
Mass used to prepare T1 (g) – STEP 2	1453.56	199.64 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

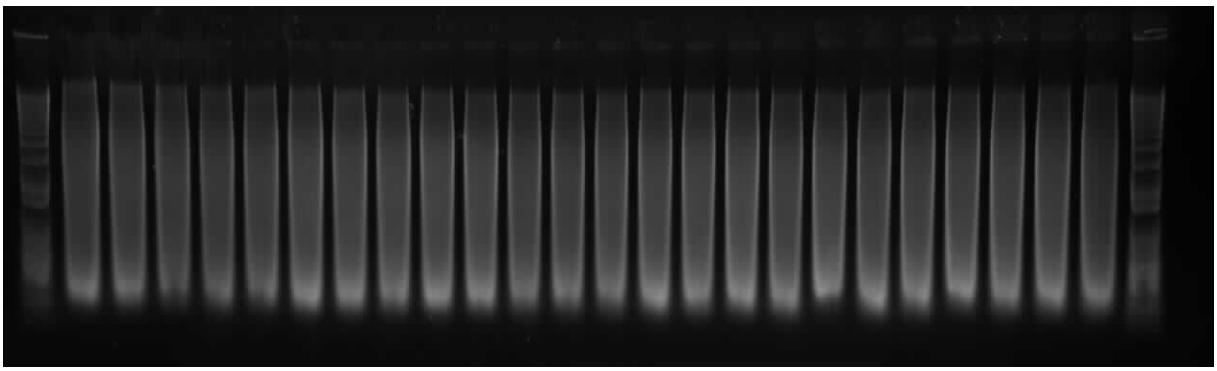


Figure 1. Agarose gel electrophoresis of genomic DNA extracted from the T1 material (lanes 2-25). The molecular marker in the first and last lane is a 1 kb Plus DNA ladder (Invitrogen, USA).

Test item T2 was prepared by mixing ground non-GM maize with T25 maize kernel flour kindly received from BASF GmbH. BASF indicated that the maize kernels were collected from plants homozygous for the T25 event and that the seed purity was > 999 g/kg. The T25 seeds used for the preparation of T2 were of the same zygosity as the official CRM used for calibration and quality control of the measurements (AOCS 0306-H10, T25 maize leaf tissue genomic DNA prepared from homozygous plants).

The absence of other crop species or GM events in the non-GM maize flour was confirmed by using pre-spotted plates for screening [7] and GM event-specific maize [8]. The maize flour was mixed with T25 flour in one step (Table 2) and filled in 5 g portions into 20 ml vials, closed under argon. The final powder had an average particle diameter of 69.4 ± 1.5 µm (k=2, n=3) with a water content of 5.5 ± 0.8 g/100 g (k=2, n=3). The amount and the quality of the DNA extracted from the T2 material using a CTAB method were verified by UV spectrometry, fluorometry (200-400 ng/µL) and gel electrophoresis (Figure 2). The results of inhibition analyses for the *hmg* target using serial dilutions passed the evaluation criteria (slope and ΔCq).

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

Characteristic	Non-GM maize	T25 maize
Type of base material	Kernels	Kernel flour
Origin	AVEVE (BE)	100 % T25 maize (delivered by BASF)
Grinding equipment	Cryo-grinding vibrating mill	/
Mixing equipment	DynaMIX CM-200	
Water content in g/100 g, mean ± U (k=2, n=3)	3.4 ± 0.4	7.1 ± 0.9
Particle diameter in µm, mean ± U[1] (k=2, n=3)	95.8 ± 17.0	142.7 ± 25.2
Mass used to prepare T2 (g)	690.54	9.45

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

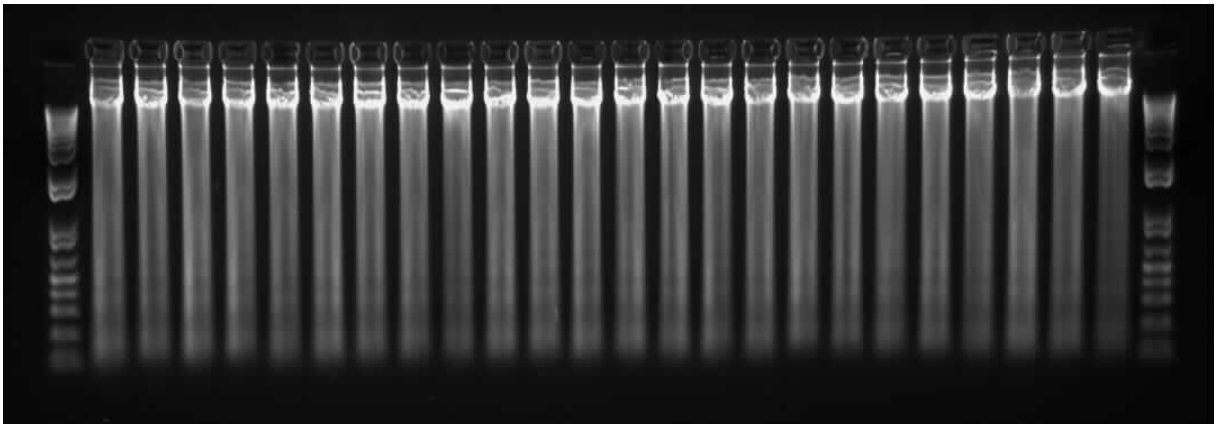


Figure 2. Agarose gel electrophoresis of genomic DNA extracted from the T2 material (lanes 2-25). The molecular marker in the first and last lane is a 1 kb Plus DNA ladder (Invitrogen, USA).

4.2 Optimisation of the GMO detection procedure for meat pâté

Initial attempts to extract genomic DNA from the meat pâté test item (T1) failed in the PCR inhibition tests. Several DNA extraction methods were tested, including different CTAB procedures suitable for DNA extraction from soybean. A routine CTAB method involving several chloroform extractions and CTAB and isopropanol precipitation did not yield high quality DNA. When used in qPCR, a variable GM content far below the expected GM % was measured. A CTAB/tip20 method produced DNA of acceptable quality based on spectrophotometric measurements, inhibition tests and qPCR. Reducing the sample intake from 200 to 100 mg further improved the results. This method consisted of a lysis step in the presence of 1 % CTAB, RNase A, proteinase K and β-mercaptoethanol, followed by 2 chloroform/octanol (24:1) extractions, CTAB precipitation and further purification of the extracts using the Genomic-tip procedure (including an additional lysis step with guanidine-HCl containing buffer G2 supplemented with RNase A and proteinase K) and a Genomic-tip 20/G column

(Qiagen, USA). The method was additionally modified by increasing the CTAB lysis time from 1 h to 3 h. The extracted DNA was tested for PCR inhibition with the lectin gene on a range of dilutions and generally passed the tests. However, similar inhibition tests using the MON89788 target often failed, which could be due to the low amount of the GM target (and total soybean) in the test item, thus resulting in large Cq values in the diluted DNA samples used. The extracted DNA had a high concentration (100-300 ng/µL based on Picogreen measurements) and acceptable absorbance (OD) ratios (260 nm/280 nm and 260 nm/230 nm; data not shown). Further modifications of the extraction procedure did not improve the quality of the DNA. For instance, an n-*hexane* treatment prior to the CTAB/tip20 method did change the qPCR results, nor did an additional NucleoSpin Food purification following the CTAB/tip20 extraction. Also NucleoSpin Food and Biotecon DNA extraction kits yielded DNA of comparable quality and GM content. A CTAB/tip20 extraction method with a reduced sample intake of 100 mg was finally chosen for further analyses (Annex 4).

Genomic DNA extracted from meat samples is known to contain compounds that may inhibit PCR, resulting from the manufacturing processes and/or incompletely removed from the matrix [9]. These compounds may interfere with the PCR by reducing the activity of the Taq DNA polymerase. Addition of a synthetic plasmid used as positive internal control has been proposed to detect PCR inhibition in DNA extracted from food or feed [10]. Furthermore, the pork meat DNA in the pâté is present in excess in the DNA extracted from T1 and the pig genome contains sequences that are at least partially identical to the primers used in the MON89788 detection method (e.g. 17 out of 20 bp of the forward primer and 17 out of 19 bp of the reverse primer; data not shown). Primer depletion may therefore be another potential source of PCR interference. Doubling the primer and probe concentration, however, did not affect the Cq values measured (data not shown).

Because of the indications of PCR inhibiting or interfering compounds in the extracted DNA, which was also evidenced by the fact that the qPCR results (using the EURL-validated method) were lower than expected (average of 1.1 m/m % MON89788), additional alternative approaches known to be less sensitive to PCR inhibition/interference were tested (Figure 3). Hot-start PCR uses an antibody-inactivated hot-start enzyme designed to minimise non-specific amplification while increasing target yield. Using JumpStart Taq ReadyMix (Merck KGaA, Germany), the same DNA extracts that yielded an average GM content of ~1.1 m/m % with the validated method using TaqMan Universal PCR Master mix (with UNG; Thermo Fisher Scientific, USA), now gave a value of ~1.5 m/m %. Similarly, using droplet digital PCR (ddPCR) known to be less affected by PCR inhibitors and also employing the hotstart technology, the average GM content measured in the same DNA extracts was ~1.4 m/m %. Also the DNA extracts obtained by the routine CTAB method (without tip 20/G purification), which gave a GM content of only ~0.5 m/m % with the validated qPCR method, measured ~1.4 m/m % using ddPCR. It is concluded that both approaches, hot-start qPCR and ddPCR, resulted in MON89788 results close to the nominal and likely true GM concentration. In contrast, when using the qPCR detection method for MON89788 validated by the EURL GMFF a negative bias of 30 to 40 % was observed for this particular test item.

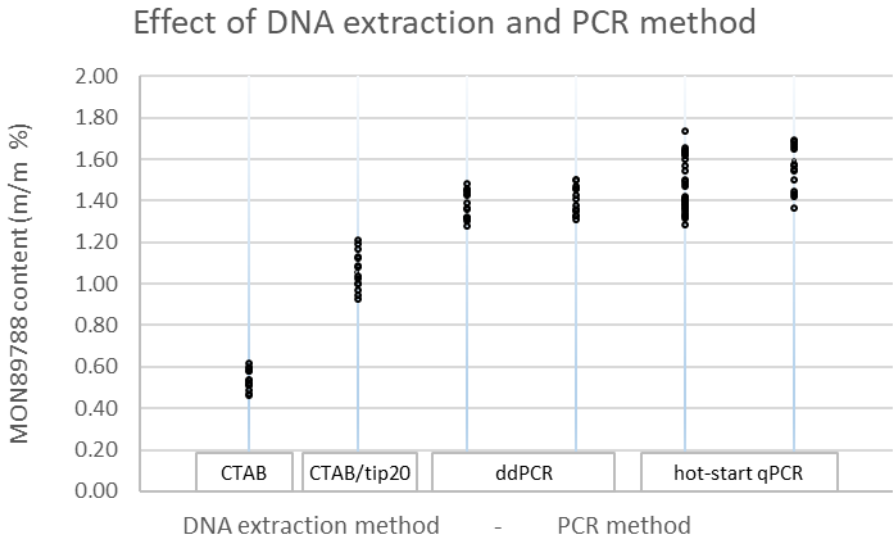


Figure 3. Optimisation of the DNA extraction and PCR procedures for MON89788 detection in meat pâté. Each method was tested on 15 DNA extracts (*N*=5, *n*=3), except the first hot-start qPCR column (5th column) which shows the results of the homogeneity study (*N*=7, *n*=5). Columns 1 and 4, and columns 2, 3 and 6, show the results obtained on identical DNA extracts.

In routine analysis, the presence of inhibiting or interfering PCR compounds in a DNA extract is not always easily detected. In particular for new and special matrices like meat-based products it is advised to check for potential inhibition with appropriate procedures and, in case of doubt, apply approaches similar as described here, to assess the validity of the measurement system used.

4.3 Homogeneity and stability

Measurements for the homogeneity and stability studies and the statistical treatment of the data were performed by the JRC for T1 and T2, using the corresponding event-specific detection methods, and taking into account the optimisation of the method as described above for T1.

The assessment of **homogeneity** was performed after the processing and bottling of the test items and before distribution to the participants. Seven sachets/bottles were randomly selected and the extracted DNA (CTAB/tip20) was analysed by hot-start qPCR in 5 replicates each (see Figure 3 and Annex 5.1). Results were evaluated according to ISO 13528:2015 [3]. The contribution from homogeneity (u_{hom}) to the standard uncertainty of the assigned value ($u(x_{pt})$) was calculated using the software SoftCRM v2.0.21 [11].

The T1 material proved to be homogeneous for the GM event (Annex 5.1).

As the T1 test item was frozen immediately after processing and the dispatching was done on dry ice, no study was conducted to assess its stability during dispatch conditions. However, the stability of the sample following thawing and continuous storage at 4 °C or after repeated freezing/thawing cycles was assessed. Five daily 100 mg samples were taken from a bottle that was either thawed and kept at 4 °C during a week, or from a bottle that was frozen again after taking a sample on 5 consecutive days. The 100 mg samples were stored at -20 °C before DNA extraction the following week. Another bottle was left at room temperature during 4h before taking a sample for DNA extraction. The qPCR results (without hot-start) showed no significant effect of freezing/thawing or any of the other storage conditions on the GM content measured. This was also communicated to the participants in the instructions letter sent following dispatch.

The homogeneity of T2 was similarly assessed and confirmed using CTAB DNA extraction and the validated T25 qPCR method (Annex 5.1).

The stability of T2 during dispatch conditions was assessed using an isochronous short-term stability study [12] involving two test samples with three replicates each (N=2, n=3) conducted over one week at +4 °C or +40 °C (3 and 7 days incubation). The measurements by qPCR were performed under repeatability conditions. The results revealed no significant influence of storage at +4 °C or +40 °C on the stability of the test item (compared to storage at -18 °C). The T2 materials could have been dispatched at room temperature, but they were dispatched in the same box together with the frozen T1 sample.

The **long-term stability** of the test items during the extended period covered by the PT round was tested by ddPCR for T1 and by qPCR for T2, analysing the GM content in bottles (N=2, n=3) stored at the normal storage temperature (-20 °C for T1, +4 °C for T2). 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 (loss/increase due to storage). No significant trend was detected at a 99 % confidence level (Annex 5.2). This stability study confirmed that T1 and T2 remained adequately stable at -20 °C or +4 °C, respectively, during the whole time period of the PT round. The uncertainty contribution to the assigned value due to instability was set to zero (u_{stab}=0) for the investigated analytes [3].

5 Assigned values and corresponding uncertainties

5.1 Assigned values

The assigned value (x_{pt}) for the mass fraction of the MON89788 event in T1 was derived from measurement results obtained by hot-start qPCR and ddPCR procedures applied to DNA extracted by the CTAB/tip20 method (Table 3). The value was close to the gravimetrically-derived expected MON89788 content (1.5 m/m %), i.e. the MON89788 mass fraction in the pâté measured corresponded to the MON89788 mass fraction per total soybean added to this matrix.

The assigned value (x_{pt}) for the mass fraction of the T25 event in T2 was derived from results reported by the JRC expert laboratories in Geel and Ispra, applying the EURL-validated qPCR method (Table 3). The value measured was considerably larger than the expected value based on the gravimetric preparation (1.3 m/m %). It was hypothesised that this may be caused by a different DNA extractability from the non-GM and GM maize flours used to prepare T2.

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	Method	Measured average per dataset ± *U* (*k*=2)	x_{pt}	u_{char}	u_{hom}	$u(x_{pt})$	σ_{pt}	$u(x_{pt})/\sigma_{pt}$
T1	MON89788	Hot-start qPCR (*N*=35)	1.47 ± 0.04	**1.47**	0.05	0.03	**0.06**	**0.37**	0.16
		Hot-start qPCR (*N*=15)	1.56 ± 0.06						
		ddPCR (*N*=15)	1.38 ± 0.04						
T2	T25	qPCR (N=35)[1]	2.23 ± 0.05	**2.36**	0.08	0.04	**0.09**	**0.59**	0.14
		qPCR (N=15)[1]	2.23 ± 0.04						
		qPCR (N=15)[1]	2.52 ± 0.04						
		qPCR (N=15)[2]	2.46 ± 0.05						

[1] Results obtained on DNA extracted by CTAB method; [2] Results obtained on DNA extracted by NucleoSpin Food method

5.2 Associated measurement uncertainties

The associated standard measurement 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 13528:2015 [3]:

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

The uncertainty u_{char} is estimated according to the recommendations of ISO 13528:2015 [3]:

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

where "*s*" refers to the standard deviation of the mean values per dataset obtained by the expert laboratories and "*p*" refers to the number of datasets.

Since $u(x_{pt})<0.3\sigma_{pt}$ for the GM event in both T1 and T2 (Table 3), the standard uncertainty of the assigned value is deemed negligible and need not to be included in the interpretation of the results [3].

5.3 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 expert judgment (Table 3).

6 Scores and evaluation criteria

Laboratory performance for the (qualitative) identification of the GM event in a test item was scored as follows: D=detected, ND=not detected, NT=test item or GM event not tested. 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 the GM event present in the test items.

For T1, in line with the prior communication to the participants, no performance evaluation is done. Instead, an estimate of deviation between the assigned value and the reported result is calculated and presented as % of the assigned value (*D%*), according to ISO 13528:2015 §9.3 [3]:

$$D\% = 100 * \frac{(x_i - x_{pt})}{x_{pt}} \ \% \qquad \text{Eq. 3}$$

For T2, the individual laboratory performance for the determination of the GM content was expressed in terms of *z* and *ζ* scores according to ISO 13528:2015 [3]:

$$z = \frac{x_i - x_{pt}}{\sigma_{pt}} \qquad \text{Eq. 4}$$

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

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.

The interpretation of the *z* and *ζ* performance scores is done according to ISO 13528:2015 [3]:

|score| ≤ 2	satisfactory performance	(green in Annex 6)
2 < |score| < 3	questionable performance	(yellow in Annex 6)
|score| ≥ 3	unsatisfactory performance	(red in Annex 6)

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*. All laboratories in this PT round reported their results with the associated uncertainty and coverage factor.

Uncertainty estimation is not trivial, therefore an additional assessment was provided to each laboratory reporting measurement uncertainty, indicating how reasonable has been their measurement uncertainty estimation. 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 13528:2015 §7.6) or, if applicable, by

formulation (ISO 13528:2015 §7.3) or than the certified measurement uncertainty associated with a certified reference material property value (ISO 13528:2015 §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 pointed out that "$u_{max,rel}$" is a normative criterion when set by legislation, however, this is not specified in the GMO legislation.

It should be understood that the reported data from participants were not $\log_{10}$-transformed prior to the performance assessment [14].

7 Evaluation of reported results

7.1 Participants

Overall, 50 NRLs from 24 EU Member States (excluding Estonia, Malta and Ireland; the latter has an agreement with Wageningen Food Safety Research in The Netherlands for GMO analysis) and 11 OCLs registered to this PT round (Table 4). Sixty participants reported qualitative and/or quantitative results for T1 and/or T2. Laboratory L35 (NRL/625) and L60 (OCL) applied only qualitative screening tests (no events identified) and L11 (OCL) did not report any result nor returned the questionnaire.

The majority of participants applied real-time PCR, while 7 laboratories reported digital PCR results for T1 and/or T2. The experimental details are presented in Annexes 6 and 7.

Table 4. Overview of participants to GMFF-21/02 by country and category

Country	Participants	NRL/625	NRL/120	OCL (not NRL)
Austria	2	2		
Belgium	3	3		
Bulgaria	2	2		
Croatia	2	2		
Cyprus	1	1		
Czech Republic	1	1		
Denmark	1	1		
Estonia	0	0		
Finland	2	1	1	
France	3	3		
Germany	16	1	13	2
Greece	1	1		
Hungary	2	1		1
Ireland	0	0		
Italy	2	1	1	
Latvia	1	1		
Lithuania	1	1		
Luxembourg	1	1		
Malta	0	0		
Netherlands	1	1		
Poland	6	3		3
Portugal	1	1		
Romania	1	1		
Serbia	1			1
Slovakia	2	2		
Slovenia	1	1		
Spain	4	2		2
Sweden	1	1		
Switzerland	2			2
Total	**61**	**35**	**15**	**11**

7.2 Laboratory results and scorings

7.2.1 Laboratory performance for GM event identification

The first step in GMO analysis of routine samples often 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.

The MON89788 GM event in **T1**, like MON87705, should react positive only in the following screening method: ctp2-CP4-EPSPS (QL-CON-00-008), while p*35S*, T*nos*, *bar* or *pat* elements, and all other screening elements are absent (see GMO-Matrix at gmo-crl.jrc.ec.europa.eu/jrcgmomatrix/).

For **T2**, the differentiation between the three possible maize events to be screened was easy as T25 was the only event containing P*35S* and *pat* and lacking T*nos*. Instead of first applying a screening strategy, the direct event-specific analysis could have been an efficient alternative approach for identifying the T25 event.

The qualitative results are summarised in Table 5, while the individual laboratory results are presented in Annex 6. Fewer laboratories analysed T1 compared to T2, which was expected as T1 was a new matrix in the EURL GMFF PT scheme and it was announced as a feasibility study, which may have demotivated some laboratories. L18 was the only laboratory who analysed T1 but could not identify the event, "*because the DNA was not suitable for [quantitative] PCR analyses due to a high practical LOQ*". The majority of laboratories demonstrated their capacity to identify the correct GM event in both matrices.

Table 5. Qualitative identification of the GM event present in T1 or T2

Test item and/or GM event tested?	Outcome	MON89788 in T1	T25 in T2
Tested	Detected (D)	51	58
	Not detected (ND)	1	0
Not tested (NT)		9	3
Total		61	61

7.2.2 Laboratory performance for determination of the GMO content

Laboratory performance for quantification of the GM event in T1 was expressed in terms of *D%* only (Annex 6). In general, all results but 2 (i.e. 96 %) deviated less than 53 % from the assigned value, which seems acceptable for this rather difficult matrix. Over 80 % of the results were lower than the assigned value and only 8 reported values (17 %) were higher. The *D%* values resulting from reported dPCR measurements were all relatively small (below 15 %), thus confirming our observations (see Section 4.2) and the fact that the x_{pt} was derived, at least partially, from dPCR data. Further details are provided in Section 7.2.6.

Laboratory performance for quantification of the GM event in test item **T2** was expressed in terms of z and ζ scores. Annex 6 presents the reported results as table and graph for each measurand. Satisfactory performance scores are highlighted in green, questionable in yellow, unsatisfactory in red. Cells were left uncoloured when the outcome could not be evaluated. The corresponding Kernel density plot has been obtained by using the software available from the Statistical Subcommittee of the Analytical Methods Committee of the UK Royal Society of Chemistry [15].

Figure 4 summarises the performance scores obtained. A total of 50 quantitative results were reported for T25 maize in T2 and have been scored. An overall satisfactory performance of 66 % (33 out of 50 reported results), expressed as z score, was obtained. Two questionable scores and a total of 15 unsatisfactory results were obtained. When taking into account the reported measurement uncertainties, 21 of the results were determined as unsatisfactory (expressed as ζ score). The unsatisfactory results obtained for T2 included 2 results significantly below the assigned value and 13 results (all using real-time PCR) between 2 and 5 times higher. The concerned laboratories were contacted for reporting the results of further investigations into the

underlying problem (root-cause analysis) and also the EURL GMFF performed additional experiments. The results are further discussed in Section 7.2.7.

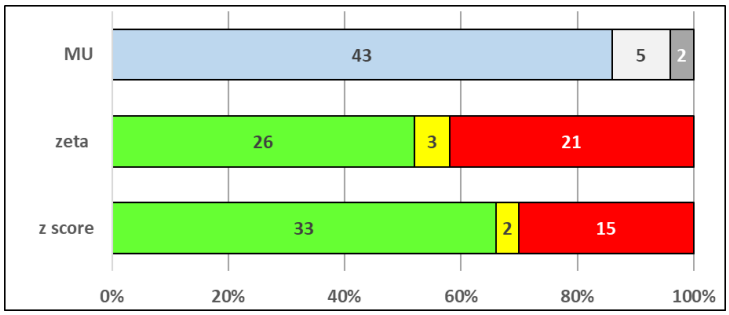


Figure 4. Overview of laboratory performance according to z and ζ scores, for the content of the T25 GM event in test item T2. Corresponding numbers of laboratories are shown in the bars. Satisfactory, questionable and unsatisfactory performance scores are indicated in green, yellow and red, respectively. 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): $u_{rel}(x_i) > \sigma_{pt,\%}$

7.2.3 Truncated values

The seven truncated values reported for T2 (> 0.025, > 0.04, > 0.045, > 0.1 and > 0.9 m/m %), are consistent with the assigned value of 2.36 ± 0.34 m/m % (k = 2). As for T1, two truncated values were reported: one consistent (> 0.045 m/m %), and one seemingly incorrect (< 0.7 m/m/ %) and well below the assigned value of 1.47 ± 0.12 m/m % (k = 2).

7.2.4 Measurement uncertainties

Nearly all laboratories having reported quantitative results provided expanded measurement uncertainties and coverage factors for T1 and T2. Only laboratory L12 (NRL/625) provided an uncertainty without a coverage factor (for T2).

The measurement uncertainties (reported for T2) were evaluated according to ISO 13528:2015 [3] (See section 6.1). Most of the laboratories (86 %) reported a realistic measurement uncertainty (Case "a" in Figure 4). Laboratory L31 (NRL/120) erroneously reported a measurement uncertainty in % of the reported value instead of in m/m %; the laboratory acknowledged this mistake afterwards by email to the PT organiser.

7.2.5 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 %). 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 because no labelling required (authorised GMO mass fraction <0.9 m/m %, if adventitious or technically unavoidable);
C<LLP Compliant because GMO falling under Regulation 619/2011 was present at <0.1 m/m % (assuming it was adventitious or technically unavoidable);
NCL Not compliant because the product should have been labelled (authorised GMO mass fraction >0.9 m/m %);
NC>LLP Not compliant because the product contains GMOs falling under Regulation 619/2011 at a mass fraction above 0.1 m/m %;

CNC Cannot conclude.

Although some testing laboratories do not usually provide such statements to their Competent Authorities when reporting their results, all laboratories should be aware of the labelling rules in the EU and should be able to properly interpret their results. As the two GM events present in T1 or T2 are both authorised in the EU, the reported range (result ± expanded uncertainty) is to be compared to the labelling threshold of 0.9 m/m % and only this Regulation applies. A few participants refer additionally to Regulation (EU) 619/2001, which is wrong as this Regulation only applies to a specific range of listed GM events, present in feed only, for which the authorisation has not been granted yet or which has expired. Hence, a product can only be compliant to Regulation (EC) 1829/2003

i). when the GM event is authorised and present at a level ≤ 0.9 % or

ii). when the authorisation is pending or has expired, the event is included in the EU GM register related to Regulation (EU) 619/2011 and it is present, in feed, at a level ≤ 0.1 m/m %.

A total of 51 and 55 participants filled in the questions regarding compliance of T1 and T2, respectively. Most of them also provided a justification for their choice among the 5 compliance options (see above). The option selected and the justification provided were evaluated.

The following assumptions were taken into account:

For test item T1

- The GM event in T1 is an authorised GM event in the EU, hence the labelling threshold to be applied is 0.9 m/m % [5].
- The content of MON89788 measured in T1 is expected to be above the labelling threshold, based on the assigned value provided and taking into consideration the measurement uncertainty ($x - U >$ Threshold).
- This material is to be considered as "Not compliant because labeling is required" (NCL) in line with Regulation (EC) No 1829/2003.
- On the basis of the reported measurement results it is also possible that $x - U \leq 0{,}9$ m/m %, in which case the sample should be considered compliant to Regulation (EC) 1829/2003 (CNL).

Table 6 summarises the statements reported for T1, taking into account the reported analytical results (or lack of results). The majority of the laboratories (36 out of 50 responses, i.e. 72 %) correctly interpreted the compliance rules based on their obtained measurement results. One participant (L37) did not understand that a value of 0.90 % ($x - U$) is to be considered compliant under this Regulation. L10 reported "not compliant under Regulation (EU) 619/2011" on the basis of an expired authorisation status of this event. However, the authorisation for MON89788 has been renewed in 2019 and the event has not been 'unauthorised' at any moment after its first authorisation was granted. Two participants did only qualitative assessment and could therefore not conclude on compliance, while another one decided that the borderline result ($x - U$ = 0.86 %) obtained would require a second analysis before concluding on compliance.

Table 6. Reported compliance statements for T1 (meat pâté)

Compliance Statement	Laboratory Measurement	Number of Laboratories	Comment
Compliant, because no labelling required	$x \pm U \leq 0.9$ m/m %	26	Correct based on the result
Compliant, under Regulation 619/2011 but <0.1 m/m %	$x \pm U \leq 0.1$ m/m %	0	
Not compliant, should have been labelled	$x \pm U > 0.9$ m/m %	20	Correct based on the result
	$x \pm U \leq 0.9$ m/m %	1	Wrong, as 0.9 % is compliant
Not compliant, under Regulation 619/2011 and >0.1 m/m %	$x \pm U \leq 0.9$ m/m %	1	Wrong because Regulation does not apply here (authorised event)
Cannot be concluded / not quantified		3	
Total no. of participants that provided a statement		51	

For test item T2

A similar evaluation of the reported compliance statements was done for T2, containing T25 maize, which was labelled as feed:

- The T25 event is an authorised GM event in the EU, hence the labelling threshold to be applied is 0.9 m/m % [5].

- Knowing that the assigned (expanded) range is 2.36 ± 0.17 (k=2) m/m %, and since 2.36 – 0.17 = 2.19 m/m %, which is above the labelling threshold (x - U ≤ Threshold)
- This material is to be considered "Not compliant because labelling is required" (NCL).

Most participants (46 out of 55, *i.e.* 84 %) made a correct compliance statement for test item T2 and provided the correct justification for their answer, *i.e.* that the event is authorised under Regulation (EC) No 1829/2003 and its content, without reasonable doubt, was above (or in 3 cases, below) 0.9 m/m % (Table 7). One participant (L16) concluded that the sample should have been labeled while the result clearly indicated it was <0.9 % and this justification was also given; probably the participant inadvertently selected the wrong statement. L04 reported 'non-compliance for labeling' and 'compliance under Regulation (EU) 619/2011', however, the latter does not apply here as explained above. L17 reported 'non-compliance under Regulation (EU) 619/2011', which is wrong for the same reason. Another participant (L29) also concluded on 'non-compliance under Regulation (EU) 619/2011', however, based on the identification of MON863 at the limit of quantification, for which the authorisation is indeed expired. The MON863 content measured was, however, 0.09 %, hence the sample would anyway pass the compliance requirements. Eight participants could not conclude on compliance, of which 7 referred to the lack of quantitative results. One participants (L61) selected both NCL (correct) and 'cannot be concluded' because of uncertainty due to the different DNA extraction from CRM and sample, different ploidy between leaf tissue (used for CRM) and seeds, and due to the unknown zygosity of sample T2. However, all these reasons do not really apply when analysing an unknown sample, as the result obtained has to be referred to the official CRM applicable, whatever its characteristics in relation to real samples. The T25 CRM is the only CRM for a maize GM event for which its certificate mentions that it is derived from homozygous maize plants, while the majority of maize seeds in the market are hybrid seeds, hence hemizygous for the GM event. However, the Commission Decision mentions the CRM from AOCS as official calibrant, hence any result has to be expressed in relation to this CRM.

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

Compliance Statement	Laboratory Measurement	Number of Laboratories	Comment
Compliant, because no labelling required	x ± U ≤ 0.9 m/m %	3	Correct based on the result
Compliant, under Regulation 619/2011 but <0.1 m/m %	x ± U ≤ 0.1 m/m %	1	Wrong because Regulation does not apply here
Not compliant, should have been labelled	x ± U > 0.9 m/m %	43	Correct based on the result
	x ± U ≤ 0.9 m/m %	1	Wrong, as U not considered
Not compliant, under Regulation 619/2011 and >0.1 m/m %	x ± U ≤ 0.9 m/m %	2	Wrong because Regulation does not apply here (L16); correct if MON863 is considered (L23)
Cannot be concluded / not quantified		8	
Total no. of participants that provided a statement		55*	

* Some participants provided more than one answer on compliance for the same sample

7.2.6 Additional information extracted from the questionnaire

The questionnaire was answered by 59 out of 61 participants. Annex 7 summarises the experimental details provided by each participant.

The majority of participants (> 68 %) reported that their laboratory was accredited in accordance with ISO/IEC 17025 for the methods used in the PT round, but other respondents have only accreditation for some of the methods used.

For the analysis on meat pâté (T1), 42 % of respondents declared to have some experience with DNA extraction from such matrix, but not necessarily in the frame of GMO control. An equal number of respondents mentioned to have no experience with this matrix. A large majority of participants used their routine method for DNA extraction from T1, sometimes with minor modifications, e.g. hexane pre-treatment to remove the fat, extended lysis time (to 3 h) and further clean-up of the DNA with a kit. Most laboratories started from 200 mg sample intake for both T1 and T2, but quite some laboratories used 500 mg or more for both T1 and T2. For both test items, about the same number of laboratories (20 and 18 for T1 and T2, resp.) applied the NucleoSpin Food kit for DNA extraction or one of the CTAB methods. Compared to previous PT tests, more laboratories tested for absence of inhibition in the DNA extracts from both T1 (25) and T2 (28) measuring the reference gene in a dilution series. In addition, 33 laboratories included two or more dilutions in the PCR experiments. Nearly all laboratories (47) reported that the DNA extracted from T1 was considered suitable for quantitative analysis, and only 1 laboratory said it was not suitable due to a high (practical) LOQ. Some other laboratories found the DNA suitable only when extracted with one of the tested extraction methods, or when sufficiently diluted (to mitigate the effect of inhibitors).

Most laboratories (47) used screening methods to limit the number of GMOs to test with event-specific methods. The most common screening strategy, used by over 40 laboratories, involved testing for p35S and tNOS, often in combination with PAT and *bar*. Also CTP2-CP4-EPSPS was often used as screening target, particularly for T1.

GM event quantification was usually done by qPCR with standard curves, while 6 laboratories used dPCR for both T1 and T2. Except in a few cases, the CRMs from AOCS were used for calibration, although different batch numbers, some of which were purchased >5 years ago, were mentioned for the T25 CRM (0306-H10 was the most recently released batch code at the time of the PT measurements and the one that should have been used). As the exact batch code number used for T25 calibration is not always provided, it is not possible to draw a relationship between the unsatisfactory results obtained for T2 and the CRM batch used. Lectin was the endogenous reference gene target for T1, and *hmg* (34) or *adh1* (18) for T2 (note that the unsatisfactory results for T2 were not related to the reference target gene used).

Interestingly, 25 laboratories used 200 or 250 ng DNA per PCR reaction for the highest calibration standard for T1, 5 laboratories used 150 ng, and 10 laboratories 100 or less than 100 ng. For the unknowns, 18 laboratories used the same DNA amount as for the standards, while 13 laboratories used less, and 9 more compared to the highest standard. Using less DNA for the unknowns could alleviate potential inhibition issues, however, will also reduce the sensitivity of the assay considering that the soybean DNA in the extracts represented only a fraction of the total (mostly meat-derived) DNA.

L12 applied a conversion factor (2) for their qPCR result obtained for T2, arguing that 'this material is heterozygous while the calibration standards of the kit used are expressed in haploid genome equivalents'. The reported result was scored as unsatisfactory (highest result reported from all laboratories) and the reasoning to multiply the result by 2 was incorrect. A few laboratories used dPCR and calculated in-house a conversion factor for T25 maize and for MON89788 soybean. The EURL GMFF has not yet issued a conversion factor for T25 because a too large variability was observed between testing laboratories and between different AOCS 0306-H batches. For the MON89788 event, the conversion factor to be applied was 0.981 ± 0.021.

7.2.7 Further investigations on the T25 analysis in test item T2

A total of 11 NRLs (OCLs were excluded), all of which obtained an unsatisfactory *z* score for the reported T25 content in T2, were contacted and asked to perform a root-cause analysis. This is in line with clause 7.10 on non-conformity of the ISO 17025:2017 standard and specified as one of the tasks of any EURL under Regulation (EU) 2017/625. The laboratory should then list the root cause(s) identified and specify the corrective action(s) necessary to prevent the non-conforming work to occur again. If possible, demonstration

of the effectiveness of these actions should be provided, e.g. in the form of new experimental results that are more in line with the expected value.

In addition to the general questionnaire that accompanied this PT round (Annex 7), the EURL GMFF collected further experimental details from all participants that had quantified T25 using a subsequent survey distributed several weeks after the PT deadline (also shown in Annex 7). The results of both surveys indicated the following:

- The DNA extraction method used had no relevant effect on the T25 content measured. Laboratories had used many different methods and there was no relationship between the method used and the measured T25 result.
- The endogenous reference method used was in most cases *hmg*, sometimes *adh1* (134 bp amplicon), with no significant effect on the T25 content measured. A few laboratories compared both reference methods and also found no significant difference in the T25 results obtained.
- Annealing temperature: most laboratories used an annealing temperature of 60 °C, L08 used 63 °C, while two laboratories used 57 or 58 °C for ddPCR.
- Different batches of the AOCS 0306-H CRM were used by the laboratories, including H3, H4, H6, H7, H9 and H10, many of which the validity was expired and should not have been used anymore. Both satisfactory and unsatisfactory results were obtained following use of different CRM batches. L14, however, reported that using the same DNA and instrumental setup (using the same qPCR 2x Master Mix plus without UNG (Eurogentec)), but replacing the H6 CRM batch with batch H10 (the batch commercially available during this PT) nearly halved the GM % measured (from 5.16 to 2.83 m/m %; L14 had originally also multiplied the result by 2 to accommodate zygosity differences, but afterwards realised this was wrong). L30 noted that the number of *hmg* copies (but not T25 copies) measured in batch H9 was larger than those in batch H10; as a result the GM content decreased from 6.09 (with H9) to 2.53 m/m % (with H10). Other laboratories that originally used an older CRM batch did not obtain a more acceptable T25 content when repeating the analysis with the latest batch (H10, now replaced by H11).
- Thermocycler used: L41 reported that switching from the MIC thermocycler to RotorGene (both from Corbett), using the same DNA and calibrant dilutions (batch H6) resulted in a roughly 4 times lower T25 % that was more in line with the expected value. L36 originally used the LightCycler (2 µL reaction volume), then switched to the CFX96 (BioRad) instrument (5 µL reaction volume): the GM % dropped 5X from 10.07 to 1.96 m/m % (also due to the use of the official CRM instead of heterozygous seeds as calibrant). In both cases, however, the instrument switch was also accompanied by a switch in master mix used.
- Master mix: at least ten laboratories with acceptable results had used the TaqMan Universal Master Mix with UNG (2 of these laboratories used type II), one laboratory used the TaqMan Fast Universal Master Mix, no UNG. Other laboratories with good results had used the Eurogentec qPCR 2X Mastermix Plus without UNG, LightCycler 480 Probes Master, Maxima probe PCR MasterMix 2x, Qiagen Multiplex no ROX, Luna® Universal Probe qPCR Master Mix, Brilliant II QPCR-Master Mix, GoTaq probe qPCR or PerfeCTaq PCR ToughMix No ROX. In contrast, among the 8 laboratories with overestimated T25 content that have reported their master mix, all had used a master mix based on hot-start technology (using an antibody-inhibited DNA polymerase), i.e. qPCR 2X MasterMix Plus without UNG, GoTaq probe qPCR, Maxima probe PCR MasterMix 2x, JumpStart TAQ Ready Mix, iQ Supermix, LightCycler FastStart DNA Master HybProbe, iTaq Universal Probes Supermix, Hot FirePol Probe qPCR Mix Plus (no ROX). Furthermore, none of these hot-start master mixes, except the Maxima Probe qPCR Master Mix, included UNG and dUTPs (see below). Note that three of the hot-start master mixes (qPCR 2X Master Mix Plus, GoTaq and Maxima) had been shown to result in both acceptable and unacceptable results in different laboratories. L52 originally used HOT FIREPol Probe qPCR Mix Plus (no ROX) and reported 4.87 % T25; after switching to TaqMan Universal 2x Master Mix the lab measured 2.83 % T25. Some laboratories, like L25, have performed many further tests using different master mixes (but not TaqMan Universal) and obtained variable, but always too high T25 percentages.

In ddPCR on the AOCS CRM (0306 batch H10 and H9) L34 recently discovered that the inclusion of dUTP in the BioRad *Supermix for probes* resulted in fewer positive copies for T25, while not affecting the number of *hmg* copies. This resulted in a GM copy number ratio of approximately 0.95 using Supermix without dUTP, but only approximately 0.55 using Supermix with dUTP. The EURL GMFF confirmed this effect of dUTP on the GM

copy number ratio. Some batch to batch (and experiment to experiment) differences in T25/*hmg* ratio were also noted. However, this negative effect on T25 amplification in the presence of dUTPs was not clearly observed for the DNA extracted from the T25 seed powder used for spiking the T2 test item (GM copy number ratio 0.84-0.90 %). It is known that dUTP is less efficiently incorporated into an amplicon compared to dTTP. The results would suggest that amplification of the T25 amplicon from the CRM DNA (but not from the seed powder DNA) would be hampered in the presence of dUTP. But these observations would still not explain the overestimated qPCR results for T2, which are all (except Maxima probe PCR MasterMix 2x) obtained with master mixes without UNG/dUTP. We observed also two PCR products with different sizes when running the ddPCR reactions obtained with the master mix without dUTPs on gel following chloroform purification. No bands were seen with the master mix with dUTPs and we have no explanation so far for this. It is however clear that non-specific amplification seems to occur.

The EURL GMFF performed further comparative qPCR experiments using different master mixes to amplify the T2-extracted DNA. No difference was measured using *TaqMan Universal Master Mix* with or without UNG/dUTP, although the amplification efficiency of the T25 method was often borderline (i.e. slope close to the lower boundary of -3.60). The BioRad hot-start master mix *SSo Advanced Universal Probes Supermix* (no UNG) resulted in a good efficiency of the T25 and *hmg* methods, but a higher T25 content in T2 (3.4 – 4.9 % in 2 different experiments, and mounting to 5.7 % on 4x diluted T2 DNA). The use of another hot-start master mix, the *JumpStart REDTaq® ReadyMix™ Reaction Mix, for High-throughput PCR of complex templates* was optimised by increasing the $MgCl_2$ concentration to 3 or 4 mM, resulting in a T25 content measured in T2 that was higher than expected (3.2 – 3.8 % for different sample dilutions). PCR products of the expected sizes were observed for both T25 and *hmg* methods after gel electrophoresis of the final PCR reaction products with JumpStart at different $MgCl_2$ concentrations and BioRad Sso master mix. However, non-specific amplification products were also observed in the T25 reactions particularly when using the BioRad master mix and with increasing $MgCl_2$ concentrations in the JumpStart master mix. Non-specific amplification was also pronounced in the *hmg* reactions when using the BioRad master mix. The results reveal an effect of the master mix, resulting in unspecific amplifications.

Further investigations will be needed to explain the remaining issues with the overestimation of the T25 content by some laboratories. Both the laboratories and the EURL GMFF are performing additional experiments for this and the results will be reported in due time.

8 Conclusions

The proficiency test GMFF-21/02 was organised to assess the analytical capabilities of EU NRLs and OCLs to determine the content of MON89788 soybean in meat pâté and T25 maize in maize flour.

All participants, except one, who tested for the presence of GM events in these test items also correctly identified the GM event in T1 and T2. As this was the first time that a meat-based food material was used, it shows that the control laboratories are competent to assess such materials for the presence of GMOs.

The overall performance of the participants for the determination of the GM event in T1 was not evaluated with performance scores, but looks fairly satisfactory. This PT round included an educational element, i.e. to consider optimisation of both the DNA extraction and the PCR part of the detection method in case of meat-based samples such as the meat pâté used here.

While most laboratories obtained acceptable *z* scores for T2, 13 laboratories significantly overestimated the T25 maize content. The experimental details provided do not allow a clear explanation for these deviations and further investigations are being carried out by the concerned laboratories and by the EURL GMFF.

With only a few exceptions, the compliance statements provided by the laboratories were considered in line with the results obtained for T1 and T2.

The general outcome of this PT round confirms once more the excellent performance of EU NRLs mandated to perform routine controls on the presence of GMOs in food and feed products on the EU market [16].

Acknowledgements

The laboratories listed hereafter are kindly acknowledged for their participation to the PT round.

Organisation	Country
AGES - Institute for Food Safety Vienna	AUSTRIA
Umweltbundesamt GmbH	AUSTRIA
CRA-W	BELGIUM
ILVO	BELGIUM
Sciensano	BELGIUM
Laboratory of SGS Bulgaria	BULGARIA
National Center of Public Health and Analysis	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
BioGEVES	FRANCE
Laboratoire de la santé des végétaux - ANSES	FRANCE
Service Commun des Laboratoires	FRANCE
Bavarian Health and Food Safety Authority (LGL)	GERMANY
CVUA-MEL	GERMANY
CVUA Freiburg	GERMANY
Federal Office for Consumer Protection and Food Safety (BVL)	GERMANY
Hessisches Landeslabor	GERMANY
Institute for Hygiene and Environment	GERMANY
Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei (LALLF) M-V	GERMANY
Landeslabor Berlin-Brandenburg	GERMANY
Landeslabor Schleswig-Holstein	GERMANY
Landesuntersuchungsanstalt fuer das Gesundheits- und Veterinärwesen Sachsen	GERMANY
LAVES-LVI Braunschweig/Hannover	GERMANY
LTZ Augustenberg	GERMANY
LUFA Speyer	GERMANY
Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft	GERMANY
Thueringer Landesamt fuer Verbraucherschutz	GERMANY
Thüringer Landesamt für Landwirtschaft und Ländlichen Raum	GERMANY
General Chemical State Laboratory (GCSL)	GREECE
Biomi Kft	HUNGARY
National Food Chain Safety Office	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	LITHUANIA
Laboratoire National de Santé	LUXEMBOURG
Wageningen Food Safety Research (WFSR)	NETHERLANDS
GIJHARS Laboratorium Specjalistyczne w Kielcach	POLAND
J.S. Hamilton Poland Sp. z o.o.	POLAND
National Veterinary Research Institute	POLAND
Plant Breeding and Acclimatization Institute NRI	POLAND
Regional Laboratory of Genetically Modified Food	POLAND
Wojewódzki Inspektorat Weterynarii w Opolu	POLAND
INIAV, I.P.	PORTUGAL
Institute of Diagnosis and Animal Health	ROMANIA
SP Laboratorija a.d.	SERBIA
Central Control and Testing Institute of Agriculture, Bratislava	SLOVAKIA

Organisation	Country
State Veterinary and Food Institute, VFI in Dolny Kubin	SLOVAKIA
National Institute of Biology	SLOVENIA
Centro Nacional de Alimentación. AESAN	SPAIN
Laboratorio de salud Pública de León	SPAIN
Laboratorio Arbitral Agroalimentario - MAPA	SPAIN
Laboratorio Central de Veterinaria	SPAIN
Livsmedelsverket (National Food Agency)	SWEDEN
Agroscope	SWITZERLAND
Federal Food Safety and Veterinary Office FSVO	SWITZERLAND

References

[1] Regulation (EU) No 2017/625 of the European Parliament and of the Council of 15 March 2017 on official controls and other official activities performed to ensure the application of food and feed law, rules on animal health and welfare, plant health and plant protection products, *Off. J. Eur. Union* L 95: 1-142 (2017).

[2] ISO/IEC 17043:2010 (2010) Conformity assessment – General requirements for proficiency testing. International Organization for Standardization, Geneva, Switzerland.

[3] ISO 13528:2015 (2015) Statistical methods for use in proficiency testing by interlaboratory comparisons. International Organization for Standardization, Geneva, Switzerland.

[4] Commission Implementing Regulation (EU) No 120/2014 of 7 February 2014 amending Regulation (EC) No 1981/2006 on detailed rules for the implementation of Article 32 of Regulation (EC) No 1829/2003 of the European Parliament and the Council as regards the Community reference laboratory for genetically modified organisms. *Off. J. Eur. Union* L 39: 46-52 (2014).

[5] Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. *Off. J. Eur. Union* L 268: 1-23 (2003).

[6] Commission Regulation (EU) No 619/2011 of 24 June 2011 laying down the methods of sampling and analysis for the official control of feed as regards presence of genetically modified material for which an authorisation procedure is pending or the authorisation of which has expired. *Off. J. Eur. Union* L 166: 9-15 (2011).

[7] Querci, M., Foti, N., Bogni, B., Kluga, L., Broll, H., Van den Eede, G. (2009) Real-time PCR-based ready-to-use multi-target analytical system for GMO detection. *Food Anal. Meth.* 2: 325-336.

[8] Gatto, F., Rosa, S., Querci, M., Kreysa, J. Development and Optimization of the GM Maize Event-Specific Pre-Spotted Plate (MePSP). *JRC Technical report* (2015).

[9] Piskata, Z., Servusova, E., Babak, V., Nesvadbova, M., Borilova, G. The quality of DNA isolated from processed food and feed via diufferent extraction procedures. *Molecules* 24: 1188 (2019).

[10] Sovová, T., Křížová, L., Drábková, L., Ovesná, J. Detection of PCR inhibition in food and feed with a synthetic plasmid. *Czech J. Food Sci.* 35: 160-164.

[11] SoftCRM, (n.d.). http://www.eie.gr/iopc/softcrm/index.html.

[12] Linsinger, T.P.J., van der Veen, A.M.H., Gawlik, B.M., Pauwels, J., Lamberty, A. (2004) Planning and combining of isochronous stability studies of CRMs. *Accred. Qual. Assur.* 9: 464-472.

[13] ISO Guide 35:2017 (2017) Reference materials - Guidance for characterization and assessment of homogeneity and stability. International Organization for Standardization, Geneva, Switzerland.

[14] Broothaerts, W., Cordeiro, F., Corbisier, P., Robouch, P., Emons, H. (2020) Log-transformation of proficiency testing data on the content of genetically modified organisms in food and feed samples: Is it justified? *Anal. Bioanal. Chem.* 412, 1129–1136.

[15] Analytical Methods Committee (2006) Representing data distributions with kernel density estimates. *AMC Tech. Br.* 4, 2. http://www.rsc.org/images/brief4_tcm18-25925.pdf.

[16] Broothaerts, W., Cordeiro, F., Robouch, P., Emons, H. (2020) Ten years of proficiency testing reveals an improvement in the analytical performance of EU National Reference Laboratories for genetically modified food and feed. *Food Control* 114: 107237.

Annexes

Annex 1: Invitation letter

EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

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

EURL European Union Reference Laboratory for GM Food & Feed

Geel, 25 August 2021
JRC.F.5/HE/wb/mt ARES(2021) 21-090

FOR THE ATTENTION OF THE
NATIONAL REFERENCE LABORATORIES (NRLS) FOR GMOS
UNDER REGULATIONS (EU) 2017/625 AND (EU) NO 120/2014

Subject: Invitation to participate to proficiency test GMFF-21/02

Dear Colleague,

Hereby, I would like to invite you for participating to the proficiency test (PT) GMFF-21/02, organised by the European Union Reference Laboratory for GM Food and Feed (EURL GMFF) in line with its mandate under Regulation (EU) 2017/625.

Participation to this PT is free of charge. Please remember that participation is mandatory for all NRLs designated under Regulation (EU) 2017/625 and recommended for NRLs nominated under Regulation (EU) No 120/2014. 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. These laboratories will have to register for this PT using the registration details provided in this letter.

Taking into account the difficult nature of the test items and tasks in this PT round, consider inviting control laboratories that will be able to report the results requested.

This PT will include two ground test materials that will be dispatched on dry ice. They are processed by the JRC and "*derived from products that are not declared as containing GM material*". Soybean is occasionally identified in meat pâté, posing a safety risk to soy-allergic people (see e.g. https://bit.ly/2WkY0tp and https://bit.ly/3kjCNIh). The testing laboratories are requested to check for the presence of GMOs and to assess the compliance of the samples with the applicable GMO legislation.

The following tasks are requested from the participants:

Test Item 1 - Meat pâté (food) (10 g fresh weight, frozen):
- Verify the presence of GM soybean in this sample;
- Quantify the (single) GM event identified and assess compliance of the sample.

Test Item 2 - Maize flour (for feed) (5 g dry weight):
- Verify the presence of the following maize events: 3272, 5307, T25;
- Quantify the (single) GM event identified and assess compliance of the sample.

Participants are requested to apply their routine approaches for GMO testing, taking care to ensure that the DNA extraction procedure used is adapted to the sample matrix and that the quality of the DNA obtained is suitable for PCR (**of particular importance for T1!**). Details on your analysis have to be reported in a questionnaire via an online EU Survey.

The quantitative results have to be reported in mass/mass %. The EURL GMFF will calculate performance scores for the reported results for T2. As this is the first time that a meat-based test item will be used in our PT

scheme, the analysis of T1 will be considered as a feasibility study. Hence, the results reported will be evaluated using the %Difference. Be aware of the existence of an appeal procedure in case you disagree with your scores.

Information on the identity of the participants in this PT will be kept confidential. However, the lab codes of the NRLs that have been designated in line with Regulation (EU) 2017/625 may be disclosed to DG SANTE for evaluation of their performance. Upon request from an NRL in a Member State, the lab codes of the official laboratories (or NRLs) within its network of control laboratories may also be disclosed to the NRL.

Please register electronically using the following link: https://europa.eu/!uqQPBp.

After registration, you are requested to return the signed registration form as scanned pdf to us by e-mail (only). Each laboratory can register only once for this PT.

The deadline for registration is set to **Sunday 12 September 2021**.

The test items will be shipped on dry ice on 28 September 2021. You are requested to inform us promptly if you have not received the samples by Friday 1 October 2021.

The deadline for submission of the results is set at 12 November 2021.

Please contact the functional mailbox JRC-EURL-GMFF-CT@ec.europa.eu for all issues related to this PT.

Yours sincerely,

e-signed

Prof. Dr. Hendrik Emons
Head of Unit

Cc: Wim Broothaerts, PT coordinator

Contact:

European Reference Laboratory for GM Food and Feed
Dr Wim Broothaerts, Project leader GMO Control
Joint Research Centre of the European Commission
Retieseweg 111, B-2440 Geel, Belgium
Tel: +32 14 57 16 12 ; JRC-EURL-GMFF-CT@ec.europa.eu

Annex 2. Test item accompanying 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, 28 September 2021

Subject: **GMFF-21/02, a proficiency test (PT) to determine the GM content in two test materials, *i.e.* meat pâté and maize flour**

Dear participant,

Thank you for participating to GMFF-21/02. Please find in this parcel (on dry ice) two test materials, T1 and T2, containing respectively 10 g and 5 g of ground sample.

Upon arrival, you should immediately store the samples as follows:

Test Item T1: Meat pâté – fresh (sachet) – **store frozen at approximately -20 °C**
Test Item T2: Maize flour – dry (bottle) – store in the fridge or freezer

Please check whether the sachet and bottle remained undamaged and frozen during transport and promptly inform us if this is not the case. There is no need to send proof of the delivery to the EURL GMFF.

Further instructions on this PT round and your individual lab code and password 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 for all issues related to this PT round.

Thank you for the collaboration in this PT round.

Yours sincerely,

e-signed

Wim Broothaerts
PT coordinator
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

EURL European Union Reference Laboratory for GM Food & Feed

Geel, 28 September 2021
JRC.F.5/WB/mt ARES(2021) 21-095

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

Reporting website	https://web.jrc.ec.europa.eu/ilcReportingWeb.
Email address used for registration:	«Contact_Email»
Password for reporting:	«Part_key»
Questionnaire	https://ec.europa.eu/eusurvey/runner/GMFF2102
Password	GMFF2102

Subject: **Instructions for GMFF-21/02, a proficiency test (PT) to determine the GM content in two test materials, *i.e.* meat pâté and maize flour**

Dear Dr «Surname»,

Thank you for participating to GMFF-21/02. In one of the following days you should receive two test materials, T1 and T2, containing respectively 10 g (wet) and 5 g (dry) of ground sample, sent on dry ice. **T1 should be stored frozen at approximately -20 °C until use, while T2 can be stored either in a freezer at -20 °C or in a fridge at approximately 4 ºC.**

It is recommended to use a **minimum sample intake of 100 mg for your DNA extractions from T1, and 200 mg for T2**, as homogeneity of the test items has been demonstrated using these amounts of sample.

The high-fat meat pâté matrix of T1 has been spiked with a small mass fraction of soybean including a single GM soybean event. To avoid DNA degradation in the wet matrix, please make sure the materials are kept at low temperature during thawing (e.g. on ice or in the fridge) and after sample taking, until addition of the lysis buffer of the extraction method used. Store the unused portion of the sample in the fridge up to 5 days or return it to -20 °C for future needs (repeated freezing/thawing seems to have no effect on the GM content measured).

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 - Meat pâté (food) (10 g fresh weight, frozen):
- Verify the presence of GM soybean in this sample;
- Quantify the (single) GM event identified and assess compliance of the sample.

Test Item 2 - Maize flour (for feed) (5 g dry weight):
- Verify the presence of the following maize events: 3272, 5307, T25;
- Quantify the (single) GM event(s) identified and assess compliance of the sample.

Participants have to apply their routine approaches for GMO testing. For the analysis of T1 (feasibility study), you are free to either apply your routine method(s) for DNA extraction or to perform further investigations in order to find a suitable (DNA extraction) method for this meat pâté matrix. 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.

The quantitative results have to be expressed in mass/mass % as outlined below and with a precision that you normally would report similar results (the value reported will be used to assess your performance score):

$$\text{mass/mass \%} = \frac{\text{mass GMO [g]}}{\text{total mass of the ingredient [g]}} \text{ x } 100$$

You are requested to pay attention to the correct estimation and reporting of the measurement uncertainty (to be expressed in m/m %, not as relative %) and coverage factor used. In addition to *z* scores (for T2), the uncertainty reported will be considered in the evaluation of the results using ζ (zeta) scores. Be aware of the existence of an appeal procedure in case you disagree with your scores.

As this is the first time that a meat-based test item will be used in our PT scheme, the analysis of T1 will be considered as a feasibility study. Hence, the results reported will be compared and possibly evaluated using the %Difference.

You can find the MILC reporting website at https://web.jrc.ec.europa.eu/ilcReportingWeb. You need the registration email address and a personal password to access this webpage; these are indicated above in the box under your address data. The system will guide you through the reporting procedure.

Don't 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 **print the completed report form, sign it 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/GMFF2102, 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).

The deadline for the submission of the results ánd the questionnaire is <u>Friday 12 November 2021</u>. 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 -20 °C (T1) or 4 °C (T2) 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

Wim Broothaerts
PT coordinator
European Union Reference Laboratory for GM Food and Feed

Annex 4. CTAB/tip20 DNA extraction method for meat pâté

Weigh 100 mg meat pate in a 2 mL microcentrifuge tube
Add 1,3 mL of CTAB BUFFER A 2% + 5 µL RNase A + 6,5 µL Proteinase K + 26 µL 2-mercaptoethanol and mix by vortexing
Incubate 3 h at 65°C, shaking at 1,400 rpm
Centrifuge 10 min at 16,000xg at RT
Transfer 800 µL of supernatant to a 2 mL microcentrifuge tube containing 1 mL of chloroform:octanol (24:1)
Mix thoroughly by inverting, incubate 5 min at RT
Centrifuge 10 min at 16,000xg at RT
Transfer 750 µL of supernatant to a 2 mL microcentrifuge tube containing 1 mL of chloroform:octanol (24:1)
Mix thoroughly by inverting, incubate 5 min at RT
Centrifuge 10 min at 16,000xg at RT
Transfer 600 µL of supernatant to a new 2 mL microcentrifuge tube containing 1200 µL of BUFFER B
Mix thoroughly by inverting, incubate 30 min at RT
Centrifuge 20 min at 16,000xg at RT
Discard the supernatant by pipetting (1 mL pipette) and conserve the pellet
Add 200 µL of 1,2 M NaCl
Incubate 5 min at 50°C, shaking at 1,400 rpm
Add 1,6 mL of G2 buffer + 2,5 µL of RNase A + 20 µL of Proteinase K
Incubate 1 h at 50ºC, shaking at 500 rpm
Centrifuge 5 min at 16,000xg at RT
Equilibrate a QIAGEN Genomic-tip 20/G column with 1 mL of QBT buffer
Apply the sample to the equilibrated Genomic-tip 20/G column by pipetting
Wash the genomic-tip 20/G column with 3 mL of QC buffer
Elute the genomic DNA with 1 mL of QF buffer (pre-warmed at 50 °C) and collect the DNA in a 2 mL tube
Add 700 µL of isopropanol to each tube, invert 10 times
Centrifuge 30 min at 10,000xg at 4 °C, discard the supernatant by pipetting (1 mL pipette)
Wash the pellet with 1 mL of 70% ethanol
Centrifuge 10 min at 13,000xg at 4 °C
Discard the supernatant by pipetting (1 mL pipette - 2 min spin - 100 µL pipette) and air-dry the pellet for 10 min
Dissolve the DNA pellet in 80 µL of TE Low Buffer preheated at 50ºC
Incubate 10 min at 50ºC, shaking at 500 rpm
Let the pellet dissolve completely overnight at RT
Store at + 4 °C (short term) or -20 °C (long term)

Annex 5: Homogeneity and stability results

5.1 Homogeneity

Homogeneity of MON89788 soybean in T1 (Hot-start qPCR)

Bottle	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5
11	1.48	1.42	1.41	1.33	1.41
30	1.40	1.37	1.64	1.62	1.34
43	1.28	1.60	1.47	1.50	1.40
54	1.62	1.50	1.33	1.63	1.38
83	1.38		1.40	1.57	1.42
110	1.36	1.63	1.36	1.73	1.39
118	1.41	1.64	1.65	1.31	1.54
Mean	1.47				
s_x	0.03				
s_w	0.13				
S_s	0				
u^*	0.03				
σ_{pt}	0.37				
$0.3 * \sigma_{pt}$	0.11				
$S_s \leq 0.3^* \sigma_{pt}$	YES				
Assessment	Passed				

Homogeneity of T25 maize in T2 (qPCR)

Bottle	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5
7	2.12	2.32	2.56	2.13	2.21
20	2.20	2.31	2.44	2.16	2.36
36	2.40	2.07	2.26	2.19	2.14
61	2.16	2.27	2.14	2.28	2.26
75	2.04	2.35	2.43	2.30	2.31
98	2.11	2.38	2.43	2.11	2.01
110	2.00	2.05	2.34	1.95	2.19
Mean	2.23				
s_x	0.06				
s_w	0.15				
S_s	0				
u^*	0.03				
σ_{pt}	0.59				
$0.3 * \sigma_{pt}$	0.18				
$S_s \leq 0.3^* \sigma_{pt}$	YES				
Assessment	Passed				

Where: σ_{pt} is the standard deviation for the PT assessment,
sX 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 [13].

All values are in m/m %

5.2 Stability

In the table below, the stability was assessed according to ISO 13528:2015 § B.5 [3].

Stability MON89788 soybean in T1 (ddPCR) (all values are in m/m %)

Weeks	Bottle no.	Replicate 1	Replicate 2	Replicate 3	Average
0	48	1.31	1.46	1.28	1.37
	50	1.39	1.32	1.43	
16	84	1.50	1.23	1.48	1.38
	90	1.33	1.48	1.26	

Slope ± 2 $SE_{(slope)}$ = 0.001 ± 2 * 0.004

Stability: passed

Stability T25 maize in T2 (qPCR) (all values are in m/m %)

Weeks	Bottle no.	Replicate 1	Replicate 2	Replicate 3	Average
0	29	2.33	2.33	2.00	2.22
	82	2.33	2.18	2.15	
20	40	2.07	2.10	2.12	2.14
	105	2.38	1.98	2.16	

Slope ± 2 $SE_{(slope)}$ = -0.001 ± 2 * 0.001

Stability: passed

Annex 6: Results and laboratory performance

- ID = GM event identification (D = detected, ND = not detected, NT = not tested, ? = no data reported)
- Compl. = Compliance statement (shown in **bold red** if considered wrong):
  CNL: compliant, no labelling required; C<LLP: compliant because <0.1 m/m % under Reg. 619/2011; NCL: not compliant because should have been labelled; NC>LLP: not compliant because >0.1 m/m % under Reg. 619/2011; CNC: cannot conclude; "--" no answer.

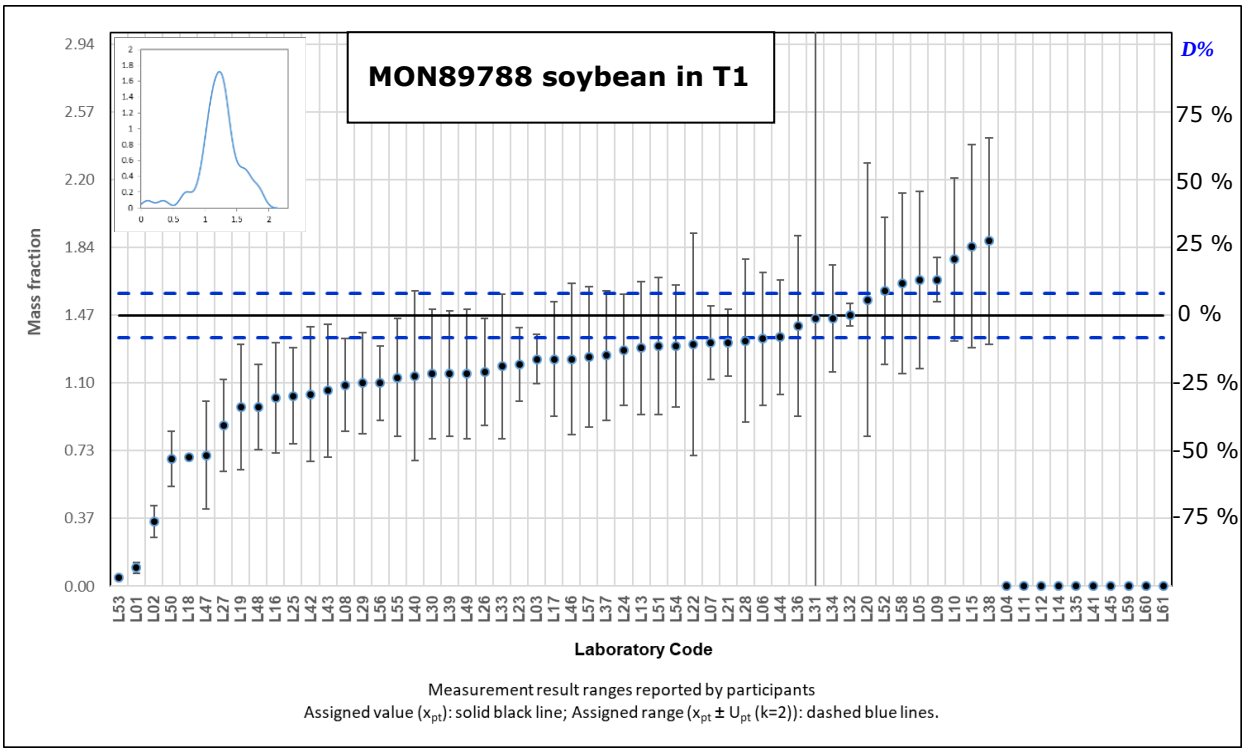
MON89788 soybean in T1

- Evaluation parameters: x_{pt} = 1.47 ; $U(x_{pt})$ = 0.12 (all values in m/m %)
- % Difference (*D%*) gives the relative difference between the reported result (x_i) and the assigned value (x_{pt}) in percentage of x_{pt}, i.e. $D\% = 100*(x_i-x_{pt})/x_{pt}$ %.

Type	Lab code	ID	=<>	x_i	$U(x_i)$	*k*	Technique	*D%*	Compl.
NRL/625	L01	D	=	0.1	0.03	2	Real-time PCR	-93.2%	CNL
NRL/625	L02	D	=	0.35	0.0875	2	Real-time PCR	-76.2%	CNL
NRL/625	L03	D	=	1.23	0.133	2	Real-time PCR	-16.2%	NCL
NRL/120	L04	NT							--
NRL/120	L05	D	=	1.66	0.48	2		13.1%	NCL
NRL/625	L06	D	=	1.34	0.36	2	Real-time PCR	-8.7%	NCL
NRL/120	L07	D	=	1.32	0.2	2.57	Real-time PCR	-10.1%	NCL
NRL/625	L08	D	=	1.09	0.25	2	Real-time PCR	-25.7%	CNL
OCL	L09	D	=	1.66	0.12	2	Real-time PCR	13.1%	NCL
NRL/625	L10	D	=	1.77	0.44	2		20.6%	**NC>LLP**
OCL	L11	?							--
NRL/625	L12	NT							--
NRL/625	L13	D	=	1.29	0.36	2	Real-time PCR	-12.1%	CNL
NRL/625	L14	NT							--
NRL/625	L15	D	=	1.84	0.55	2	Real-time PCR	25.4%	NCL
OCL	L16	D	=	1.02	0.3	2	Real-time PCR	-30.5%	CNL
OCL	L17	D	=	1.23	0.31	2	Real-time PCR	-16.2%	NCL
NRL/625	L18	ND	<	0.7			Real-time PCR		CNL
NRL/625	L19	D	=	0.97	0.34	2	Real-time PCR	-33.9%	CNL
NRL/625	L20	D	=	1.55	0.74	2	Real-time PCR	5.6%	CNL
NRL/120	L21	D	=	1.32	0.18	2	dPCR	-10.1%	NCL
NRL/120	L22	D	=	1.31	0.6	2	dPCR	-10.8%	CNL
NRL/120	L23	D	=	1.2	0.2	3.18	Real-time PCR	-18.2%	NCL
NRL/625	L24	D	=	1.28	0.3	2	Real-time PCR	-12.8%	NCL
NRL/625	L25	D	=	1.03	0.26	2	Real-time PCR	-29.8%	CNL
NRL/625	L26	D	=	1.16	0.29	2	Real-time PCR	-21.0%	CNL
NRL/625	L27	D	=	0.87	0.25	2	Real-time PCR	-40.7%	CNL
NRL/625	L28	D	=	1.33	0.44	2	Real-time PCR	-9.4%	CNL
NRL/625	L29	D	=	1.1	0.275	2	Real-time PCR	-25.1%	CNL
NRL/625	L30	D	=	1.15	0.35	2	Real-time PCR	-21.7%	CNL
NRL/120	L31	D	=	1.45	43	2	Real-time PCR	-1.2%	NCL
NRL/120	L32	D	=	1.47	0.06	3.18	dPCR	0.2%	NCL
NRL/120	L33	D	=	1.19	0.39	2	Real-time PCR	-18.9%	CNL
NRL/625	L34	D	=	1.45	0.29	2	dPCR	-1.2%	NCL
NRL/625	L35	D							--
NRL/625	L36	D	=	1.41	0.49	2	Real-time PCR	-3.9%	NCL
NRL/625	L37	D	=	1.25	0.35	2	Real-time PCR	-14.8%	**NCL**
NRL/625	L38	D	=	1.87	0.56	2	Real-time PCR	27.4%	NCL
NRL/625	L39	D	=	1.15	0.34	2	Real-time PCR	-21.7%	--

Type	Lab code	ID	=<>	x_i	$U(x_i)$	k	Technique	D%	
NRL/625	L40	D	=	1.14	0.46	2	Real-time PCR	-22.3%	CNL
OCL	L41	NT							--
OCL	L42	D	=	1.04	0.364	2	Real-time PCR	-29.1%	CNL
NRL/625	L43	D	=	1.06	0.36	2	Real-time PCR	-27.8%	CNL
OCL	L44	D	=	1.35	0.31	2	dPCR	-8.0%	NCL
NRL/625	L45	NT							--
NRL/625	L46	D	=	1.23	0.41	2	Real-time PCR	-16.2%	CNL
NRL/625	L47	D	=	0.71	0.29	2	Real-time PCR	-51.6%	CNL
NRL/120	L48	D	=	0.97	0.23	2	Real-time PCR	-33.9%	CNL
NRL/625	L49	D	=	1.15	0.35	2	Real-time PCR	-21.7%	CNL
NRL/625	L50	D	=	0.69	0.15	2	Real-time PCR	-53.0%	CNL
NRL/625	L51	D	=	1.3	0.37	2	Real-time PCR	-11.4%	NCL
NRL/625	L52	D	=	1.6	0.4	2	Real-time PCR	9.0%	NCL
NRL/120	L53	D	>	0.045			Real-time PCR		CNC
NRL/120	L54	D	=	1.3	0.33	2	Real-time PCR	-11.4%	NCL
NRL/120	L55	D	=	1.13	0.32	2	Real-time PCR	-23.0%	CNL
OCL	L56	D	=	1.1	0.2	2	Real-time PCR	-25.1%	CNL
NRL/120	L57	D	=	1.24	0.38	2.16	dPCR	-15.5%	CNC[1]
OCL	L58	D	=	1.64	0.49	2	Real-time PCR	11.7%	NCL
OCL	L59	NT							--
OCL	L60	NT							--
NRL/120	L61	D							--

[1] The laboratory mentioned that although the sample would be compliant based on the results obtained, in practice a second sample would be analysed before concluding on compliance.



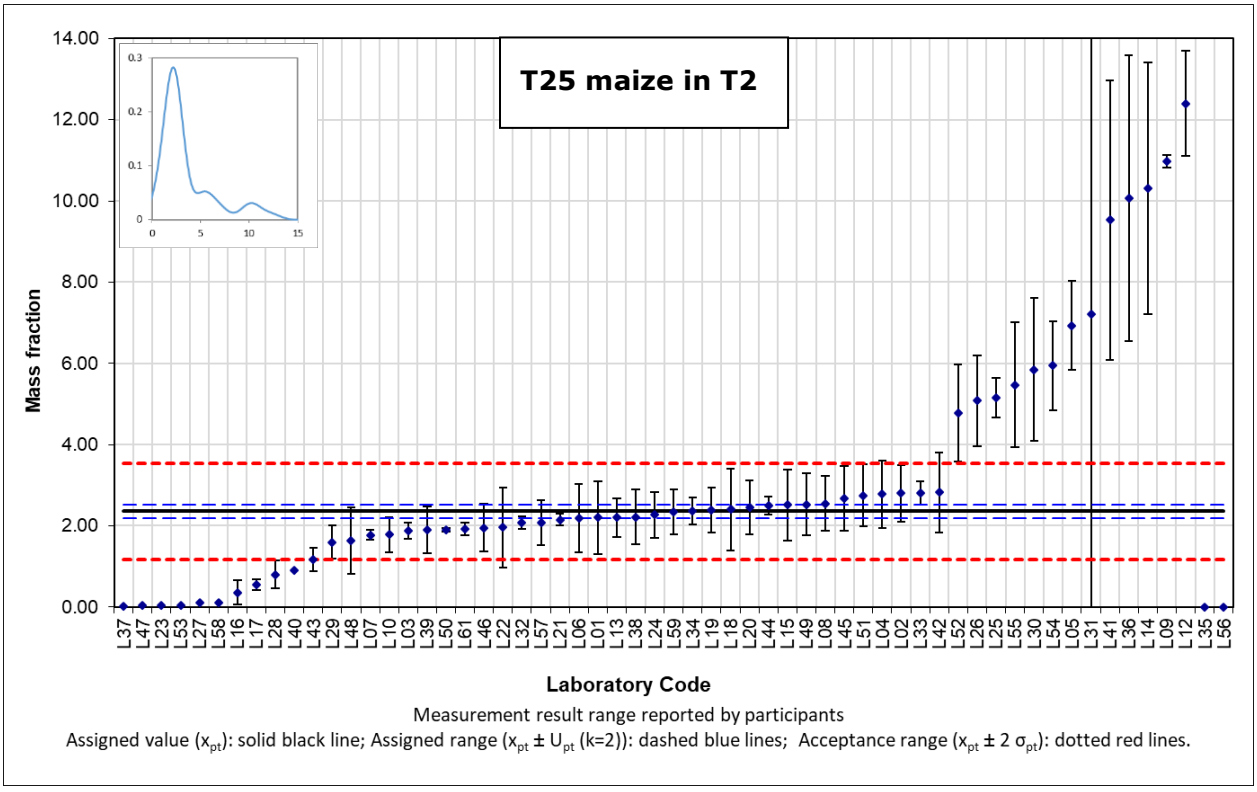
Upper left: kernel density distribution

T25 maize in T2

- Evaluation parameters: x_{pt} = 2.36 ; $u(x_{pt})$ = 0.17 ; σ_{pt} = 0.59 (all values in m/m %)
- 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: $u(x_{pt,rel}) \leq u(x_i) \leq \sigma_{pt}$; b: $u(x_i) < u(x_{pt})$; c: $u(x_i) > \sigma_{pt}$

Type	Lab code	ID	=<>	x_i	$U(x_i)$	k	Technique	z score	ζ score	MU	Compl.
NRL/625	L01	D	=	2.2	0.9	2	Real-time PCR	-0.27	-0.35	a	NCL
NRL/625	L02	D	=	2.8	0.7	2	Real-time PCR	0.75	1.23	a	NCL
NRL/625	L03	D	=	1.87	0.202	2	Real-time PCR	-0.83	-3.73	a	NCL
NRL/120	L04	D	=	2.78	0.83	2	Real-time PCR	0.71	0.99	a	NCL, **C<LLP**
NRL/120	L05	D	=	6.93	1.1	2	Real-time PCR	7.75	8.22	a	NCL
NRL/625	L06	D	=	2.19	0.84	2	Real-time PCR	-0.29	-0.39	a	NCL
NRL/120	L07	D	=	1.77	0.12	2.57	Real-time PCR	-1.00	-6.16	b	NCL
NRL/625	L08	D	=	2.55	0.67	2	Real-time PCR	0.32	0.55	a	NCL
OCL	L09	D	=	10.97	0.15	2	Real-time PCR	14.60	76.78	b	NCL
NRL/625	L10	D	=	1.78	0.44	2		-0.98	-2.46	a	NCL
OCL	L11	?									
NRL/625	L12	D	=	12.4	1.29	1.73	Real-time PCR	17.03	13.38	a	NCL
NRL/625	L13	D	=	2.2	0.48	2	Real-time PCR	-0.27	-0.63	a	NCL
NRL/625	L14	D	=	10.31	3.1	2		13.48	5.12	a	NCL
NRL/625	L15	D	=	2.51	0.88	2	Real-time PCR	0.26	0.34	a	NCL
OCL	L16	D	=	0.36	0.3	2	Real-time PCR	-3.39	-11.65	c	**NCL**
OCL	L17	D	=	0.55	0.14	2	Real-time PCR	-3.07	-16.61	a	**NC>LLP**
NRL/625	L18	D	=	2.4	1	2	Real-time PCR	0.07	0.08	a	NCL
NRL/625	L19	D	=	2.38	0.55	2		0.04	0.07	a	NCL
NRL/625	L20	D	=	2.46	0.66	2	Real-time PCR	0.17	0.30	a	NCL
NRL/120	L21	D	=	2.15	0.14	2	dPCR	-0.35	-1.92	b	NCL
NRL/120	L22	D	=	1.96	0.98	2	dPCR	-0.68	-0.80	a	NCL
NRL/120	L23	D	>	0.045			Real-time PCR				CNC
NRL/625	L24	D	=	2.27	0.57	2	dPCR	-0.15	-0.30	a	NCL
NRL/625	L25	D	=	5.15	0.48	2	Real-time PCR	4.73	10.98	a	NCL
NRL/625	L26	D	=	5.08	1.12	2	Real-time PCR	4.61	4.81	a	NCL
NRL/625	L27	D	>	0.1			Real-time PCR				CNC
NRL/625	L28	D	=	0.8	0.34	2	Real-time PCR	-2.64	-8.23	a	CNL
NRL/625	L29	D	=	1.6	0.4	2	Real-time PCR	-1.29	-3.50	a	NCL, **NC>LLP**
NRL/625	L30	D	=	5.85	1.76	2	Real-time PCR	5.92	3.95	a	NCL
NRL/120	L31	D	=	7.22	43	2	Real-time PCR	8.24	0.23	c	NCL
NRL/120	L32	D	=	2.08	0.15	3.18	dPCR	-0.47	-2.91	b	NCL
NRL/120	L33	D	=	2.81	0.28	2	Real-time PCR	0.76	2.77	a	NCL
NRL/625	L34	D	=	2.37	0.33	2	Real-time PCR	0.02	0.06	a	NCL
NRL/625	L35	D									CNC
NRL/625	L36	D	=	10.07	3.52	2	Real-time PCR	13.08	4.38	a	NCL
NRL/625	L37	D	>	0.025			Real-time PCR				
NRL/625	L38	D	=	2.22	0.67	2	Real-time PCR	-0.24	-0.40	a	NCL
NRL/625	L39	D	=	1.9	0.57	2	Real-time PCR	-0.78	-1.55	a	
NRL/625	L40	D	>	0.9			Real-time PCR				CNC
OCL	L41	D	=	9.53	3.44	2	Real-time PCR	12.16	4.16	a	NCL
OCL	L42	D	=	2.82	0.987	2	Real-time PCR	0.78	0.92	a	NCL
NRL/625	L43	D	=	1.17	0.28	2	Real-time PCR	-2.02	-7.30	a	CNL
OCL	L44	D	=	2.49	0.22	2	dPCR	0.22	0.95	a	NCL

Type	Lab code	ID	=<>	x_i	$U(x_i)$	k	Technique	z score	ζ score	MU	Compl.
NRL/625	L45	D	=	2.67	0.8	2	Real-time PCR	0.53	0.76	a	CNC
NRL/625	L46	D	=	1.95	0.59	2	Real-time PCR	-0.69	-1.33	a	CNC
NRL/625	L47	D	>	0.04			Real-time PCR				
NRL/120	L48	D	=	1.63	0.815	2	Real-time PCR	-1.24	-1.75	a	CNL
NRL/625	L49	D	=	2.53	0.76	2	Real-time PCR	0.29	0.44	a	NCL
NRL/625	L50	D	=	1.9	0.04	2	Real-time PCR	-0.78	-5.35	b	NCL
NRL/625	L51	D	=	2.75	0.77	2	Real-time PCR	0.66	0.99	a	NCL
NRL/625	L52	D	=	4.78	1.2	2	Real-time PCR	4.11	4.00	a	NCL
NRL/120	L53	D	>	0.045			Real-time PCR				CNC
NRL/120	L54	D	=	5.94	1.09	2	Real-time PCR	6.07	6.50	a	NCL
NRL/120	L55	D	=	5.47	1.54	2	Real-time PCR	5.28	4.02	a	NCL
OCL	L56	NT									
NRL/120	L57	D	=	2.08	0.55	2.06	dPCR	-0.47	-1.00	a	NCL
OCL	L58	D	>	0.1			Real-time PCR				CNC
OCL	L59	D	=	2.34	0.56	2	Real-time PCR	-0.03	-0.07	a	NCL
OCL	L60	NT									
NRL/120	L61	D	=	1.92	0.16	2	Real-time PCR	-0.74	-3.80	a	NCL, CNC



Upper left: kernel density distribution

Annex 7: Results of the questionnaires

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.

Please select which test items were analysed by your laboratory

T1		Answers	Ratio	T2		Answers	Ratio
Yes		52	88.14%	Yes		58	98.31%
No		7	11.86%	No		1	1.69%
No Answer		0	0%	No Answer		0	0%

Are the methods used within the scope of accreditation of your laboratory under ISO/IEC 17025:2017?

T1		Answers	Ratio	T2		Answers	Ratio
Yes		41	69.49%	Yes		40	67.8%
No		6	10.17%	No		4	6.78%
Partially		8	13.56%	Partially		14	23.73%
Not applicable		4	6.78%	Not applicable		1	1.69%
No Answer		0	0%	No Answer		0	0%

Further explanations regarding work not done under accreditation:

In the absence of certified reference material, quantification is only possible with a high level of measurement uncertainty. The PCR efficiencies are very different between target and reference. In the case of an official sample, this result would not be reported without further examination using dPCR. At the moment we are only setting up the dPCR. In reality, we would have hired another laboratory to do the checking.
Our accredited matrix is only feed not food, meat pate is not a feed.
For soybean we are accredited only on raw products (seeds and grains), not food and feed
For T1 we have accreditation for screening methods and for detection but not quantification of event MON89788. For T2 we have accreditation for screening methods.
Identification and quantification of the considered events are not yet under accreditation. Flexible scope is expected ASAP (for 2022 at the latest).
T25 method is accredited only as a qualitative one
T25 is not accredited
Methods not accredited but in verification.
Droplet digital PCR is not yet accredited
For T1, a second DNA extraction method was used (this has not yet been formally completely verified in the laboratory)
Events 3272, 5307, T25 and screening elements te-9, PAT and BAR that have been used are not within the scope.
Detection is done under accreditation but not the quantification
3272 and 5307 accreditation pending. T25 only detection accredited
Detection of T25/ 3272/5307 are not accredited as a method but screening elements are.
T1 (meat paté) was understood as being food and not feed, thus was not performed
Our accreditation is only for qualitative methods 3272 and 5307. We don't have accreditation for method T25
tE9, pat
Quantification T25
T1: accreditation applies not for food; T2: method not verified yet
The sample matrix is out of the scope of our laboratory.
T1 was not analysed due to changes of responsibilities for the analysis of GM soybean in control laboratories in our region from 2022 forward.
Quantification was done by another lab

Please explain why T1 and/or T2 was not analysed

		Answers	Ratio
a) The sample matrix is out of the scope of our laboratory		4	6.78%
b) The methods are not validated in our laboratory		2	3.39%
c) We could not obtain sufficient good quality DNA suitable for further analysis		0	0%
d) Appropriate Certified Reference Material was not available		2	3.39%
e) Primers, probes, or other reagents were not available (in time)		1	1.69%
f) We tried but our analysis failed		0	0%
g) Other practical constraints (instrument broken, no personnel, etc.)		1	1.69%
h) Other reason		2	3.39%
No Answer		51	86.44%

Did your lab have previous experience with DNA extraction from the meat pate matrix (T1)?

Yes	No
25	26

Other comments:

Our laboratory had no experience with DNA extraction from the meat pate matrix. Our flexible scope of accreditation covers only plant matrix.
No, only with canned pet feed (e.g. for dogs) in terms of animal species identification
Yes, but not for GMO analysis purpose and only for qualitative PCR analyses.
We have experience from different food samples, not necessarily meat pate
Yes, but not for GMO analysis
Yes, we have some experience. We have about 10-15 similar samples (liver pate, luncheon meat, hamburger meat) per year.
Yes, as part of the molecular biological differentiation of animal species.

Did your lab apply its routine methods for DNA extraction from the meat pate (T1) or performed further investigations to optimise the extraction method for this matrix? Please provide as much details as possible.

Routine method	Optimised method
45	5

Further comments:

We have used the DNEASY MERICON FOOD (Qiagen) Kit, which is under routine use for food matrixes. No further investigation has been performed.
Our lab applied our routine method - NucleoSpin Food (NSF) kit (Macherey-Nagel) - extract B In addition, two modifications of the standard protocol were applied and tested: - extending the lysis time to 3 hours and additional purification of the DNA extract using the Wizard DNA Clean-Up System (Promega) - extract A and - applying the n-Hexane before NSF extraction - extract D. Moreover, we applied also Nucleospin DNA Lipid Tissue kit (Macherey-Nagel) designed for gDNA isolation from lipid-rich samples of human/animal origin but we obtain very low yield - extract C. The results of three DNA extracts (A, B and D) were used for the calculation of the final result.
We apply our routine methods (CTAB based) to all food matrices. If the controls (e.g. inhibition control) do not meet expectations, modifications are applied on a case-by-case basis (use of various enzymes, e.g. amylase).
Routine method, hexane extraction step before CTAB DNA isolation used for fatty products.
Routine methods were applied. Some minor modifications were done (e.g. fat layer was omitted).
We performed investigations to optimise the extraction method. 1. Literature search - the method that we use (NucleoSpin® Food Macherey Nagel kit) is suitable for DNA extraction from pate; 2. we extracted DNA from 1 g of the sample and extended incubation time.
CTAB extraction followed by clean-up with KIT Macherey- Nagel

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	12	1	2	25	3	10	0
T2	13	0	4	39	1	1	0
No Answer	45	58	54	17	56	49	59

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	2	2
CTAB method with 2% CTAB in lysis buffer	12	13
CTAB + Maxwell 16 Food, Feed, Seed	5	7
NucleoSpin Food	20	18
NucleoSpin Plant	0	3
GeneSpin	3	3
Promega Wizard	3	2
Qiagen DNeasy Plant	0	0
Qiagen DNeasy Mericon Food	2	3
Biotecon Foodproof	2	2
SDS	0	2
Fast ID Genomic DNA	0	0
Generon Ion Force	0	0
Eurofins DNAExtractor cleaning column	0	0
Promega Wizard DNA clean-up resin	2	3

Qiagen QIAQuick	1	3
Qiagen Genomic-Tip 20/G	0	0
NucleoSpin gDNA clean-up	1	1
Other	13	8

Further details on DNA extraction method used:

Chloroform for meat paté
innuPREP Plant DNA Kit of Analytic Jena with innuPure C16 automatic
T 1 and T 2: Incubation time for DNA lysis was doubled to 3 h.
Nucleomag Food on KingFisher Duo Prime Purification System.
NucleoMag(R) Food; Macherey-Nagel using a Kingfisher flex
1nd extraction for T1: CTAB 2nd extraction for T1: guanidine hydrochloride based lysis followed by Wizard column extraction and resin clean-up
SureFood(R) Prep Advanced in T1 and after purification with QIAquick (R)PCR purification kit
In the case of the pâté, we observed inhibition with the CTAB method and performed the CTAB Maxwell extraction as an alternative, and there were no problems with this.
Hexane extraction step before CTAB DNA isolation used for fatty products.
T1: any fat layer or fat particles in the lysate were omitted from DNA extraction
Modified Qiagen DNeasy Blood & Tissue kit
RNase A was added and 30 min incubation at 65°C; Proteinase K was added and again 30 min incubation at 65°C; the fat layer visible on top of the extracts after these additions, was not included further in the downstream extraction steps.
SureFood®Prep Advanced Kit; 5 x 200 mg sample extracted and pooled for analysis
Additional purification was done with the use of hexane.

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

Answer	**T1**	**T2**
No	4	5
We performed a PCR inhibition test on a reference gene target prior to the analysis	25	28
We performed a PCR inhibition test on a GM gene target prior to the analysis	3	3
We analysed two or more dilutions of the DNA and compared the results	33	33
An internal positive control was added to the unknown samples	4	4
Other	3	2

Further clarification on the approach used for DNA quality analysis and the outcome:

We determined the DNA concentration with NanoDrop 2000
DNA quality was assessed by spectrophotometry . The ratios A260/A280 and A260/A230 were in the expected range. The concentration of the extracts was high. All samples needed to be diluted to 20 ng/uL.
Our laboratory used its routine methods for control quality of DNA (measurement of concentration of DNA, purity of DNA, integrity of DNA) and control of inhibition of DNA
We measured Absorbance and check the relation A260/230 and A260/280. Both have values > 1.7
T1: for qPCR and ddPCR two dilutions were analysed: 1:10 and 1:20 (DNA in H2O); inhibition questionable in qPCR but not in ddPCR T2: for qPCR and ddPCR two dilutions were analysed: 1:10 and 1:20 (DNA in H2O); inhibition questionable in qPCR but not in ddPCR
We always evaluate the amplification curves for the samples
Approach described in the guide Verification of analytical methods ... Annex 2: Evaluation of DNA- extraction method (inhibition test)
We run several dilutions of the transgenic and endogene target and compare the delta Ct, inhibition is observed at ΔCt >0.5
Double stranded DNA concentration and quality measurements were done to optimize template amounts in qPCR analyses. The LOD value calculations and inhibition tests were performed for T1 and T2 as instructed by ENGL guidelines. Everything seemed OK - the LOD values were in usual range.
DNA fragmentation was analysed on a microchip; 260/280 nm ratios were calculated with spectrophotometer
We check that the ratios OD260/280 and OD260/230 are acceptable [Nanodrop].
For DNA quality testing, we follow the QS-strategy instructions for our laboratory. This includes information on the use of controls and necessary activities if the controls do not produce the expected result.
We checked the dilution of the two duplicates with a biophotometer and the results were similar.
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: lectin, T2 *hmg*). The ΔCq-value was assessed for PCR inhibition (expected ΔCq +/-0.5).
4-fold dilution series (undiluted + 1:4, 1:16, 1:64) of DNA extract was tested for each test item with the endogenous reference target PCR (Lectin resp. *Hmg*)
Two dilutions were analyzed with a reference gene and the expected dCT value compared to the expected one

Do you consider the DNA extracted from T1 as suitable for quantitative PCR analyses? Did you see any indications that this is not the case? Please consider that this is a feasibility study, therefore, you are invited to provide as much details as possible.

DNA from T1 was not suitable for quantitative PCR analyses because of high pLOQ
We considered the extracted DNA replicates suitable for the quantitative PCR analysis verifing the absence of inhibition in the inhibition test
We did it in the same way as routine samples, we had enough DNA, DNA quality was satisfaying, we got results with correct parameters, so we considered that our results might be correct.
We consider DNA extracted from T1 to be suitable for quantitative PCR analyses. Using the NucleospinFood Kit, we obtained a higher concentration of isolated DNA compared to the plant matrix. This is the first DNA isolation from the meat pate matrix.
Basically, the CTAB method seems fit for purpose. However, Quantifications were performed in "standard" and fast" modes (using Applied Biosystems master mixes, respectively Universal master mix, no amperase for standard mode and Fast Advanced master mix for fast mode). A highest coherence between different dilutions (Delta Ct ~1 between PCR using respectively 40 ng and 20 ng, especially for the GM target) was observed when using Fast conditions / master mix than the one observed when using Standard/Universal conditions. For this reason, only the fast conditions results were used for reporting quantitative results on test item 1.
We have noticed a high variability of measurement results (RSDr = 23.79 %) but still less than 25 % (MPR). The variability was highest (24.16 %) in the results for DNA extract B (where result was lowest) and lower in results for DNA extracts A and D which were obtained using a modified NSF protocol (18.68 % and 17,21 %, respectively). It seems that NSF method and its modifications used in the test appear to be suitable for DNA extraction from this type of matrix (meat paté).
Slight inhibition in undiluted DNA. No inhibition observed for dilutions used in further quantitative analysis.
Basically we consider DNA extracted from T1 as suitable for quantitative PCR analysis. However we initially quantified MON 89788 with qPCR and obtained inconsistent results between DNA isolates [0,79 % and 1,34 % (m/m)]. We then used carrier DNA (hering sperm) at a final concentration of 1 ng/µl in the PCR reaction but inconsistent results persisted. In ddPCR however we analysed T1 DNA with and without carrier DNA and got consistent results with both approaches. Therefore we submitted ddPCR results for T1 (MON 89788).
The DNA-extraction of this sample was performed by to separate persons on different days with different DNA-extraction methods. The quantitative as the Cq-values if the qualitative results are very good comparable. Yes, I consider that the DNA extracted from T1 was suitable for quantitative analyses.
We see signs of inhibition, but not so strong that it should stop quantification
The extracted DNA had very high concentration and optimal OD parameters (A260/280 = 1.9, A260/230 = 2). As we performed screening analysis, we performed inhibition test on the reference gene lectin and no inhibition was observed. No inhibition was observed for both reference and event-specific targets when the event was quantified.
Extracted DNA was tested on Biospectrometer Basic (Eppendorf). A 260 = 0.988 (resulting in 494 ng/ul) and 1.044 (resulting in 521.9 ng/ul)The A260/A280 index was 1.88. The dilution test did not indicate inhibition.
We did observe slight deterioration in inhibition test signal (in 1:64 and 1:256 dilutions) in T1, but when the quantitative assays were performed, both replication rounds gave results of similar range. I cannot be concluded weather the DNA quality/amount of soy in sample was causing the variation in analysis results, or if it was due to the standards used.
The extracted DNA was suitable for quantitative analysis (purity, no inhibition)
DNA was suitable: high yield, low degradation, no inhibition
DNA extracted from T1 with our CTAB classic method was not suitable for qualitative and quantitative analyses, a strong inhibition was observed (obligatory inhibition control) applying our CTAB protocol without any modifications. DNA extracts from CTAB-Maxwell did not show any inhibition and were used for conducting PCR.
After many dilutions of DNA, we got enough DNA extraction for quantitative PCR analyses.
DNA was measured on Nanodrop after isolation and was found suitable for quantitative PCR analysis
We consider the DNA extracted from T1 as suitable for quantitative PCR analyses. We measured high DNA concentrations, but only a small amount comes from the analyte (soybean event). For PCR the total amount of DNA in the reaction has to be considered. The necessary dilution of the DNA extract could lead to problems concerning the LOQ or LOD.
Pure DNA extracts were not suitable as clear inhibition in the qPCR was observed; we used 1:4 dilutions for all qPCR reactions.
Yes, the DNA extracted from T1 with NucleoSpin Food kit was suitable for quantitative PCR analyses.

If screening methods were used, please indicate the results (presence or absence).

Screening target	T1: present	T1: absent	T2: present	T2: absent
P35S	0	40	26	0
tNOS	0	41	1	25
PAT	0	36	20	1
BAR	0	23	0	15
CP4-EPSPS	4	2	0	3
Ctp-CP4-EPSPS	1	1	0	1
Ctp2-CP4-EPSPS	30	0	0	16
Cry1Ab/Ac	0	18	0	9
Cry1Ab	0	0	0	0

pFMV	19	1	0	9
pNOS	0	3	0	1
t35S	0	0	0	0
nptII	0	5	0	2
p35S-pat	0	7	5	0
tE9	15	0	1	5
Other	3	5	2	4

Comments

tOrf23
For T1: CV127 was additional screened and was negative. For T2, screening was not performed as the request was to verify the presence of three events. Events were directly identified.
AgroBorder 1: T1/T2 absent, AgroBorder 2: T1 present, T2 absent; PCsVMV-pat: T1/T2 absent; T1: Multiplex Event Specific PCR: GTS 40-3-2 absent, MON 89788 present; A 5547-127: absent; A2704-12 absent; DP356043-5 absent; DP305423-1 absent; CV 127-9 absent; MON88701 absent
Other: tNOS+nptII
T2 was not screened, only identification of the 3 events was performed
We used for T1: PSP plate for gm soy (Product code: PSP-SOY-1-A); For T2: PSP plate for gm maize (PSP- MAI-1-A).
In T2 we saw a weak signal for NPTII (in 1 out of 4 replicates, with Cq value 42,19) which we deemed as "negative"
T1: Further Event-specific methods were used to identify the GMO
To identify the GMO in T1, a soybean event-screening was done using tetraplex qPCR event-screening methods. To identify the GMO in T2, the 3 maize events were screened using singleplex qPCR methods.
SAMS absent, CV127 absent, 87705 absent, 87708 absent, 87769 absent, MON89788 (RR2) present.

Which quantification approach was used?

Quantification approach	**T1**	**T2**
Standard curve method (2 calibration curves)	42	43
Delta Cq method (one calibration curve)	1	3
Digital PCR	6	6
No quantification done	2	6

Select the calibrant(s) used for the standard curve, if applicable.

Calibrant	**T1**	**T2**
CRM from JRC-Geel	0	1
CRM from AOCS	43	42
Other	3	6

Details on CRM used

	CRM	**Answers**
T1	0906-B	12
	0906-B2	6
	0809-A	1
	From Eurofins GMOQuant (HR) Event MON89788 Soy kit	1
	Plasmid calibrants with both targets in 1:1 ratio	1
T2	0306-H	7
	0306-H2	3
	0306-H4	1
	0306-H6	1
	0306-H7	2
	0306-H9	2
	0306-H10	4
	CRM for T25 obtained directly from Bayer CropSciences	1
	From Eurofins kit GMOQuant T25 corn	2
	Old grain material obtained from the distributor	1
	Plasmid calibrants with both targets in 1:1 ratio	1
	Inhouse	1

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

	Taxon-specific endogenous reference target	**Answers**
T1	Soybean lectin	49
T2	Maize *hmg*	34
	Maize adh1	18
	Maize invertase	1

What was the total amount of DNA used per PCR reaction well (in ng/well) for T1? (Number of answers)

ng DNA	For the calibration standards (S1)	For the unknowns (samples)
<100 ng	6	6
100 ng	5	10
150 ng	6	7
200 ng	21	15
250 ng	3	2
>250 ng	1	5
Not known	6	4
No Answer	11	10

Provide details of any conversion factor used to convert your results for T1 and T2.

	Conversion factor	Answers
T1	No conversion factor used	17
	0.98(1)	7
	0.933	1
	0.956	1
T2	No conversion factor used	24
	Results x2[a]	1
	0.838	1
	0.856	1
	0.97	1

[a] "According to kit manufacturer, calibration standards are expressed as % HGE. Thus, the results obtained for the sample "maize flour" (heterozygous) were multiplied by conversion factor of 2."

Please provide further details on the quantitative analysis performed for T1, e.g. is the value reported the average of a number of replicates or the average of results obtained using different DNA extraction methods, were all values obtained comparable or did you observe differences when applying different extraction methods (please provide as many details as possible), etc.

Number of DNA extraction methods used	Number of replicates	Answers
One DNA extraction method	"Several" replicates	14
	2 extracts (replicates)	16
	3 extracts	2
	4 extracts	2
	5 extracts	1
	6 extracts	1
Two different DNA extraction methods/modifications	2 extracts each	1
Three different DNA extraction methods/modifications	3 extracts each	1

Additional comments and suggestions

T2: GM maize T 25 is a rare case for a GM maize being homozygous. Most GM maize events are heterozygous. Results for T 25 obtained by ddPCR (cp/cp %) that were reprted therefore did not have to be converted by a conversion factor into m/m %. Nevertheless, it was assumed that the T 25 DNA detected in the unknown sample is also homozygous for the transgene as the CRM from AOCS.
The exact amount of DNA is not needed when the result is in %ww. Just a need for use amounts of DNA resulting in Cq values within the range of the calibration curve.
There are not enough measurements and quantifications of T25 in our lab at the moment, therefore, an expanded relative uncertainty U of 50 % is assumed. Based on these results the labelling of the product cannot be reliably demanded. Under these circumstances a second sample would be analyzed and further measurements should be undertaken to precisely calculate the MU.
T25 maize event is rarely (not to say never) detected/identified and quantified in routine GMO analysis in our lab/country. Because of this lack on data from routine analyses, we had to order genomic DNA certified reference materials from AOCS, which took a very long time to be delivered (as usual). Also, the calibration curve for the T25 maize system was not optimal and even with repetitions of QN runs for this event, we did not succeed in optimizing the curve (efficiency of amplification NOK). Huge amount of work for an event that is never analyzed in routine (at least not being identified in any routine sample in 2019, 2020 and 2021 so far). We believe analyzing events that are frequently detected in routine GMO analyses, in PT rounds, makes much more sense for labs in charge of official GMO analyses.
Interesting round, thank you!

Results of the additional questionnaire regarding T25 maize analysis, sent after the reporting deadline

Labcode in GMFF-21/02	Which brand of PCR instrument did you use (for real-time or digital PCR)?	Did you use the official, validated method QT-EVE-ZM-011?	Details on deviations from the validated method (except those specified below)	Which mastermix did you use (brand and type)?	Did the mastermix contain dUTP and UNG?	Hotstart	Which primers and probe concentrations did you use (note: the T25 protocol recommends 400 nM for primers and 200 nM for the probe)?	What was the annealing temperature used?	Total amount of sample DNA used per PCR tube (and method used to measure this)?	Which calibrant did you use?	Further details, observations or comments
L01	ABI 7500 FAST (ADH) + Biorad CFX96 (T25)	Yes, with modifications	No initial 50°C step (no UNG in mastermix) + 40 cycles instead of 45	Eurogentec qPCR 2X MasterMix Plus without UNG (ref RT-QP2X-03WOU+)	No	yes	0.4 µM for primers and 0.2 µM for probe (as in protocol)	60°C (as in protocol)	Not determined. ADH copy number around 20 000 copies/well in the dilution used for quantification.	AOCS 0306-H4	Reference gene : QT-TAX-ZM-001
L02	Real-time	Yes	HMG was used as a taxon-specific reference target	TaqMan Universal Master mix II (Applied Biosystems)	dUTP Yes, no UNG	no	400 nM for the primers T25 and 200 nM for the probe	60 °C	200 ng (NanoPhotometer Implen)	AOCS 0306-H10	
L03	real-time PCR system ABI 7900HT	No	GMOQuant LibertyLink Corn kit -Eurofins GeneScan	provided by the kit (contain dUTP and no UNG)	Yes	?	provided by the kit and the concentration is not specified	60 C	200ng	Other	provided by the kit; made from a mixture of homozygous T25 corn and non-GMO corn (1% w/w)
L04	LC480	Yes, with modifications	60'' annealing	LightCycler 480 Probes Master	No	yes	500 nM / 150 nM in target and reference-Master	60 °C	Not determined. On Friday we could measure the amount of DNA with a fluorimeter.	Other	It is exciting to see how the quantitative results for T25 will turn out. That is why we are reporting a value here that we would not otherwise have published without further protection. At an ISTA PT in 2009, the results were very diffuse, precisely because of the lack of certified reference material.
L06	real time PCR Quant Studio 7 Life Thechnologies	Yes, with modifications	See comments	Universal Master Mix (2X TaqMan®) Life Technologies	Yes	no	for T25 we use the concentration indicated in the protocol (400 nM and 200 nM), for the HMG gene we use for primers 300 nM and for probe 180 nM indicated in the article Paternò et al.	60°C	200 ng	AOCS 0306-H10	for T25 detection we use the validated method of JRC QT-EVE-ZM-011 without any modification. For detection of endogenous gene we use HMG gene according to the method indicated in the following article: Paternò A, Marchesi U, Gatto F, Verginelli D Quarchioni C, Fusco C, Zepparoni A, Amaddeo D, Ciabatti I - Finding the joker among the maize endogenous reference genes for genetically modified organism (GMO) detection - J Agric Food Chem. 2009 Dec 9;57(23):11086-91
L08	real-time PCR - Roche LightCycler 2.0	Yes		Applied Biosystems TaqMan Master Mix with UNG	Yes	no	400 nM for primers and 200 nM for the probe	63 C	spectrofotometer; max 200 ng	AOCS 0306-H9	
L09	real-time PCR AB 7500	Yes		GoTaq qPCR Master Mix von Promega	No	yes	yes 400 nM and 200 nM	60 °C	150 ng/25 µl	AOCS 0306-H10	
L10	AnalytikJena - qTower3G	Yes	We used hmg as the reference species specific gene	Maxima probe PCR MasterMix 2x - Thermo Scientific (K0261)	Yes	yes	The same as the T25 protocol	60 °C	100 ng - Nanodrop	AOCS 0306-H7	
L12	Applied Biosystems 7900HT real time PCR System	Yes, with modifications	We have used the Eurofins kit "GMOQuant event T25 corn" (Cat number 5125208501, LOT: 21872104) which is "based to the EURL-GMFF validated method".	Eurofins kit "GMOQuant event T25 corn" (Cat number 5125208501, LOT: 21872104)	Don't know	?	We have used the Eurofins kit "GMOQuant event T25 corn" (Cat number 5125208501, LOT: 21872104)	60°C	100 ng	AOCS 0306-H5	We have used the adh1 gene for maize reference gene. Eurofins informed us that the calibration standards was DNA from AOCS 0306-H5 reference material.
L13	QuantStudio 12K Flex Real-Time PCR Instrument	No	See comments	TaqMan™ Universal PCR Master Mix by Thermo Fisher Scientific, Catalog number: 4304437	Yes	no	T25 1-5' (500 nM); T25 1-3' (500 nM); T25-2-Taq (200 nM)	59,9 °C	100 ng of maize DNA; STN EN ISO 21571 - Annex B; Methods for quantification of extracted DNA; B.1 Basic UV spectrophotometric method	AOCS 0306-H6	We used the method by STN EN ISO 21570, C.9 Construct specific method for the quantitation of maize line T25 DNA using real-time PCR, because it is accredited according to ISO 17025. Instead of SSIIb taxon specific gene target sequence, we used the maize hmg reference gene.
L14	Real-Time PCR QuantStudio 7Flex (Life Technologies)	Yes		qPCR Mastermix plus without UNG (Eurogentec)	No	yes	400nM for primers and 200nM for the probe	60°C	160ng (Quant iT PicoGreen dsDNA Assay Kit (Invitrogen))	AOCS 0306-H6	no
L15	BioRad CFX96	Yes, with modifications	hmg insteed of adh used as taxon-specific reference gene	Qiagen Multiplex no rox	No Yes	yes	400 nM for primers and 200 nM for probe	60oC	100 ng/well; measured with fluorometer	Other	calibrant was DNA purchased from Bayer Crop Science Cert. No. REF-010/2006, Lot# 32RMM00200

L17	THERMOFISHER QUANTSTUDIO 5	Yes		TAQMAN UNIVERSAL PCR MASTERMIX THERMOFISHER	Yes	no	According to the method without modifications	60	150ng	AOCS 0306-H3	As mentioned in the previous report we didn´t use MRC 0% T.25, but MRC 0% MON810.
L19	Applied Biosystems, QuantStudio 5 (bloc 0.2ml)	Yes, with modifications	Results given by our laboratory were based on measurements using 1) the validated method (except for UNG content and step) and 2) the same PCR assay but used in "fast" mode (protocol by defaur on QS5 : 1x20 sec @ 95°C +45 x (1 sec @ 95°C +20 sec @ 60°C).	Standard protocol: TaqMan™ Universal PCR Master Mix, no AmpErase™ UNG (Applied Biosystems™); fast mode : TaqMan™ Fast Advanced Master Mix (Applied Biosystems™)	No	no	see QT-EVE-ZM-011	60°C	100 ng to 200 ng / PCR (DNA concentration measured with nanodrop One)	AOCS 0306-H10	
L20	QuantStudio 5 (Applied Biosystems)	Yes, with modifications	For relative quantification, a hmg (79-bp) maize-specific reference system was used instead of Adh1 (135-bp).	TaqMan Universal PCR Master Mix (Applied Biosystems, Cat. No. 4304437)	Yes	no	T25 specific system: primers 400 nM, probe 200 nM (according to the protocol)	60 °C	50, 75, 100, 150 ng; DNA concentration was measured fluorometrically.	AOCS 0306-H9	
L22	Bio-Rad QX100 for ddPCR	Yes, with modifications	For the transgene T25 the primers/probe from method QT-EVE-ZM-011 were used. For the maize reference gene hmg instead of adh1 was used. The primers/probe were taken from the method CRL-VL-25/04VR. Reason for this deviation is that by experience adh1 under certain conditions shows non-satisfying results in gm maize quantification. Published comparative experimental date show that hmg is an universally appropriate gene for maize reference gene detection and GMO quantification.	ddPCR Supermix for Probes (No dUTP); Bio-Rad No 186-3024	No	yes	for T25: 400 nM for the primers and 200 nM for the probe for hmg: 300 nM for the primers and 160 nM for the probe	60 °C	< 100 ng; measured with fluorescence dye	AOCS 0306-H6	qPCR was initially performed (ABI QuantStudio 7; TaqMan Universal Master Mix, 4318157, Applied Biosystems) but results were not submitted because very inconsistent results were obtained. Quantification results with T2 varied from 2,86 % up to 4,73 % for T 25 for unknown reasons. 100 % AOCS material also gave inconsistent results ranging from 68,8 % to 117,5 %. ddPCR results, however were constantly around 2,0 - 2,1 %. Note: Some other German laboratories reported deviations in T25 quantification with ddPCR using different Bio-Rad mastermixes either containing dUTP or not containing dUTP. For the latter with 100 % AOCS-0306-H10 material the correct zygosity of 0,95 was observed whereas mastermixes containg dUTP showed zygosity values of around 0,55.
L25	Mx3000 Strategene	Yes	no	2 x JumpStart Taq ReadyMix (Sigma)	No	yes	400 nM for primers 200 nM for probe	60 degrees	Not measured 2x diluded, 3x diluded from the DNA purification	AOCS 0306-H10;AOCS 0306-H4	There was absolutely nothing looking strange!
L26	AB StepOne™ Real-time PCR System	Yes, with modifications	Taxon target HMG, MaximaTM Probe qPCR Master Mix (Thermo scientific)	MaximaTM Probe qPCR Master Mix (Thermo scientific)	Don't know Yes	yes	As recommended 400 nM for primers and 200 nM for the probe	60 °C	~20-25 ng	AOCS 0306-H10	
L28	real-time PCR ABI StepOnePlus	Yes		TaqMan Real time PCR Master Mix Diagenode DMMM-2X-A300	Yes	no	T25: 400/400/200; Zm Adh 200/200/200	60°C	100 - 50 - 25 ng measured by Nanodrop	AOCS 0306-H1	our laboratory had as a deviating z-score (-2.64) we have measured lower % of T25. We have experienced problem with obtaining good calibration curve with the delta Ct method complying with the acceptance criteria, as the dilution points are prepared by mixing GM and WT material. We think this is one of the cause of this result. As a long term solution we are going to use standard curve method for quantification instead of deltaCt, replacing ZmAdh with hmg.
L29	ABI 7300 real-time PCR system	No	https://publications.jrc.ec.europa.eu/repository/handle/JRC84152 Event-specific method for the quantitation of Maize line T25 using real-time PCR from 14 june 2005; Corrected version 1 - 28/08/2013; CRL VL0804VP-Corrected version 1 - 30/11/2011.	Luna® Universal Probe qPCR Master Mix	Yes	yes	for T25: 400 nM for primers and 200 nM for the probe	60 degrees Celsius	200ng/5mkl (spectrophotometer)	AOCS 0306-H10	
L30	real-time PCR	Yes		iTaq Universal Probes Supermix, BioRad, cat No 1725134	No	yes	Primers: 0.1 umol/L; Probe: 0.05 umol/L	60 oC	200 ng (Spectrometer)	AOCS 0306-H9	

L31	BioRad CFX96 (real-time)	Yes, with modifications	Our reaction volumes are 20µl, we have validated /verified them in our laboratory	BioRad - iQ-supermix (2x)	No	yes	primers 300 / probes 200 (this method has been working during validations, no idea why not working in PT)	60	1st round, 2nd round: ~100 ng (A and B sample), 3rd round: 100 ng (A sample) & 150 ng (B sample) (all measured with dsDNA BR Qubit kit)	AOCS 0306-H10	fresh patch of reference material
L32	BioRad QX200	No		ddPCR™ Supermix for Probes (Bio-Rad #186-3010)	Yes		500 nM primers and 100 nM probes	60°C	not known, DNA was used diluted and in a 1:10 dilution	None	
L34	QX200	Yes	none	Bio-Rad ddPCR Supermix for Probes (no dUTP)	No		Primer/Primer/Probe = 400/400/200 nM	60 °C	Sample T2 = 200 ng; Positive Control = 5 to 200 ng; Method: PicoGreen Fluorimetry	None	Also measured in parallel using realtime PCR: similar result (2,3 %m/m); Mix: TaqMan Universal PCR Master Mix 2x Part No 4304437; same Oligos and temperature, same sample DNA concentration; calibrant: AOCS 0306-H9 (dCt method)
L36	First try: LightCycler ; second try: CFX96	Yes, with modifications	hmg was used as taxon specific method and we used two standard curves (for first and second try)	First try: LightCycler FastStart DNA Master HybProbe (Roche) Second try: Taqman Universal PCR Master Mix (2x) (Applied Biosystems)	No/Yes	yes	Primers : 0,5 µM Probe : 0,4 µM	60°C	not applied	AOCS 0306-H10	The AOCS 0306-H10 was used for the second try. For the first one we used seeds that we received from a german lab some years ago.
L39	ABI PRISM ViiA7, Applied Biosystems	Yes, with modifications	20ul reaction, 4ul DNA template, HmgA reference gene.	TaqMan Universal master mix (2x), Applied Biosystems	Yes	no	900nM primers and 200nM probe	60C	For DNA quantity and quality check we have used qPCR for HmgA and T25 amplicon: dilution series including 3 points with 3-fold dilutions.	AOCS 0306-H10	
L42	Real Time PCR Stratagene Mx 3000P and Real Time PCR Aria Mx (Agilent technologies)	Yes	No deviations	Agilent technologies, Brilliant II QPCR-Master Mix	Yes	yes	Primers and probe are according the T25 protocol	60 degrees of Celsius	170ng/ul	AOCS 0306-H10;AOCS 0306-H9	Master mix contain dUTP but not UNG concerning question above
L44	QX200 droplet digital PCR System (Bio-Rad)	Yes, with modifications	See comments	ddPCR Supermix for Probes (no dUTP), Cat.No. 186-3024, Bio-Rad	No		400 nM for primers, 200 nM for the probe (for both T25 GM-target and hmg taxon-target assays)	57.1 °C	100 ng (UV-absorbtion at 260 nm using the NanoDrop One instrument)	AOCS 0306-H9	For DNA extraction the NucleoSpin Food Kit (Macherey-Nagel, Cat.No. 740945) was used As maize reference gene assay the hmg taxon-target was used (QT-TAX-ZM-002), not the adh1 gene. The probes of T25 GM-target and hmg taxon-target are both quenched with BHQ1 (not TAMRA). The probe of the hmg taxon-target is HEX-labeled (not FAM), to run the hmg assay together with the T25 target assay in a duplex ddPCR with FAM/HEX fluorophores. The ddPCR reaction volume is 20 uL (not 25 uL). The thermocycling was adapted for ddPCR with the QX200 System (95°C for 10 min, then 45 cycles with 94°C for 30 sec, 57.1°C for 60 sec, then 98°C for 10 min).
L45	Applied Biosystems™ QuantStudio™ 3	No	Our method : Characterization and event specific-detection by quantitative real-time PCR of T25 maize insert (Collonnier et al., 2005)	TaqMan™ Fast Universal PCR Master Mix (2X), no AmpErase™ UNG	No	yes	T25 protocol (QT-EVE-ZM-011) recommends 400 nM = 400 nmol/L for primers ASPM1 : 5'-TCA ATT GCC CTT TGG TCT TCT GA-3' (300 nM) ASRevPM1 : 5'-TAC GAC ATG ATA CTC CTT CCA C-3' (300 nM) ASFBP3 : 5'-FAM-TCA TTG AGT CGT TCC GCC ATT GTC G-Eclipse Dark Quencher-3' (200 nM)	60°C	We want 80000 copies / well. Our extracts are quantified by fluorimetry (PicoGreen) and we make a dilution in TEx0.1 to reach 80000 copies / well. Our results with Hmga range are 88693 and 87863 copies.	AOCS 0306-H9	
L46	Real Time PCR 7500, Applied Biosystems	Yes, with modifications	different reference Gene (hmg)	TaqMan Universal PCR Master Mix, Applied Biosystems	Yes	no	ref. gene: ZM-F, ZM-R, ZM P [FAM/TAM] - 300nM, 300nM, 160nM; T25: MLD143, MDB551, TM016 [FAM/TAMRA] - 400nM, 400nM, 200nM	60	200ng, spectrofotometric and Annex 3 from doi: 10.2760/645114: Production of intermediate concentrations od positive material	AOCS 0306-H9;Other	AOCS 0306-C4 - T25 DNA absent

L48	real-time PCR QuantStudio 5	Yes, with modifications	we used the primer KVM182 and KVM183 300 nM each	GoTaq probe qPCR from Promega	No	yes	we used the primer KVM182 and KVM183 300 nM each, the T25 PCR was applied with no modifications	The protocol was as described, 60°C annealing temperature	apprx. 300 ng	Other	we used an inhouse plasmid which contains the target pcr amplicons in a 1:1 ratio. The correctness of the sequences was verified by Sanger sequencing and determination of the size of the plasmid. We did not use any conversion factor for the reported % value of the gm content of the sample. We have also measured AOCS DNA AOCS 0306-H2+, T25 homozygous, Certified value ng/µg >999,9 against the plasmid standard. These results indicate that the targets in the AOCS material are not represented in a 1:1 ratio. The T25 target is rather less than 70 % of the adh target. Nevertheless did we not apply a conversion factor to our results.
L50	ViiA7 Applied Biosystems	Yes	-	TaqMan Universal PCR Master Mix Applied Biosystems	Yes	no	400 nM for primers and 200 nM for the probe	60 oC	30-35 ng/µl in tube x 5µl = 150 -175 ng in PCR (NanoDrop Spectrophotometer ND-1000)	AOCS 0306-H10	-
L51	ABI 7500	Yes	1. The calibration curve is on five points. The first point of the calibration curve (S1) has 3,6% maize T25 DNA in a total of 200 ng of maize DNA. Standards S2 to S4 are to be prepared by serial dilutions (dilution factor 2 for samples S2-S4). Stardard S5 is to be prepared by dilution for sample S4- dilution factor 5. 2. For the maize (Adh1) probe is labelled with VIC at its 5'-end and MGBNFQ at 3'-end.	TaqMan Universal Master-Mix II, Applied Biosystems	dUTP Yes, no UNG	no	Primer and probe concentrations for T25 and Adh1 are according to the validated method QT-EVE-ZM-011.	60°C	Total amount of sample DNA used per PCR: 60 ng Nucleic Acids quantification and analysis: Eppendorf Biophotometer	AOCS 0306-H10	
L52	QuantStudio™ 5 , Applied Biosystems™	Yes, with modifications	Hmg instead of Adh1 as reference gene, shorter initial denaturation (15 sec. instead of 10 minutes according to instructions of used mastermix_see below)	HOT FIREPol Probe qPCR Mix Plus (no ROX), 5X (SOLIS BIODYNE)	No		400 nM (primers) and 200 nM (probe)	60°C	65 ng (method Quantus™ Fluorometer_Quantifluor ONE dsDNA System_Promega)	AOCS 0306-H10	
L57	digital PCR with QX200, BioRad	Yes, with modifications		ddPCR SuperMix for Probes (no UTP), BioRad	No		concentration of primer / probe: 900nM / 250nM	58°C	80 to 100 ng per reaction, NanoDrop2000	AOCS 0306-H10;AOCS 0306-H6	
L59	AriaMx, Agilent	Yes, with modifications	maize taxon specific method: QT-TAX-ZM-002 (hmgA gene, ISO 21570)	Quantabio: PerfeCTaqPCR Tough Mix NoROX	No	yes	400 nM for primers, 200 nM for the probe	60 °C	180 ng for T25-PCR, 90 ng for hmg-PCR, DNA concentrations were measured photometrically, a DNA dilution series was tested by T25-and hmg-PCR prior to quantitation to determine the optimal dilution for quantitation	AOCS 0306-H7	

GETTING IN TOUCH WITH THE EU

In person

All over the European Union there are hundreds of Europe Direct information centres. You can find the address of the centre nearest you at: https://europa.eu/european-union/contact_en

On the phone or by email

Europe Direct is a service that answers your questions about the European Union. You can contact this service:

- by freephone: 00 800 6 7 8 9 10 11 (certain operators may charge for these calls),

- at the following standard number: +32 22999696, or

- by electronic mail via: https://europa.eu/european-union/contact_en

FINDING INFORMATION ABOUT THE EU

Online

Information about the European Union in all the official languages of the EU is available on the Europa website at: https://europa.eu/european-union/index_en

EU publications

You can download or order free and priced EU publications from EU Bookshop at: https://publications.europa.eu/en/publications. Multiple copies of free publications may be obtained by contacting Europe Direct or your local information centre (see https://europa.eu/european-union/contact_en).

KJ-NA-31103-EN-N

The European Commission's science and knowledge service

Joint Research Centre

JRC Mission

As the science and knowledge service of the European Commission, the Joint Research Centre's mission is to support EU policies with independent evidence throughout the whole policy cycle.

EU Science Hub

ec.europa.eu/jrc

@EU_ScienceHub

EU Science Hub - Joint Research Centre

EU Science, Research and Innovation

EU Science Hub

Publications Office of the European Union

doi:10.2760/68798

ISBN 978-92-76-53107-4