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# European technical guidance document for the flexible scope accreditation of laboratories quantifying GMOs

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**Contact information**
Stefanie Trapmann
Address: Institute for Reference Materials and Measurements, Retieseweg 111, 2440 Geel, Belgium
E-mail: stefanie.trapmann@ec.europa.eu
Tel.: +32 (0)14 571 714
Fax: +32 (0)14 571 548

http://irmm.jrc.ec.europa.eu/
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# European technical guidance document for the flexible scope accreditation of laboratories quantifying GMOs

S. Trapmann[1)], C. Charles Delobel[2)], P. Corbisier[1)], H. Emons[1)], L. Hougs[3)], P. Philipp[4)], M. Sandberg[5)], M. Schulze[6)]

1) European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (EC JRC-IRMM), Geel, BE
2) European Commission, Joint Research Centre, Institute for Consumer Protection and Health (EC JRC-IHCP), Ispra, IT
3) Danish Veterinary and Food Administration, Ringsted, DK
4) Service Commun des Laboratoires (SCL) Laboratoire de Strasbourg, Illkirch-Graffenstaden, FR
5) Livsmedelsverket, Uppsala, SE
6) Rötgesbüttel, DE

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Disclaimer

Certain commercial equipment, instruments, and materials are identified in this paper to specify adequately the experimental procedure. In no case does such identification imply recommendation or endorsement by the European Commission, nor does it imply that the material or equipment is necessarily the best available for the purpose.

## *Contents*

## *Glossary*

| | |
|---|---|
| bp | basepair |
| cp | copy |
| Cq | quantification cycle |
| CRM | certified reference material |
| CTAB | Cetyl trimethyl ammonium bromide |
| EA | European co-operation for Accreditation |
| EC | European Commission |
| ENGL | European Network of GMO Laboratories |
| EU | European Union |
| EURL-GMFF | European Union Reference Laboratory for Genetically Modified Food and Feed |
| GM(O) | genetically modified (organism) |
| GUM | Guide to the Expression of Uncertainty in Measurements |
| IEC | International Electrotechnical Commission |
| IHCP | Institute for Health and Consumer Protection |
| ILC | interlaboratory comparison (also referred to as interlaboratory study) |
| IUPAC | International Union of Pure and Applied Chemistry |
| IRMM | Institute for Reference Materials and Measurements |
| ISO | International Organization for Standardization |
| JRC | Joint Research Centre |
| LOD | limit of detection |
| LOQ | limit of quantification |
| LLP | low level presence |
| MP | microlitre pipette |
| MPR | minimum performance requirements |
| MU | measurement uncertainty |
| $N$ | number of samples |
| $n$ | number of measurement replications on the same sample |
| PCR | polymerase chain reaction |
| qPCR | quantitative (real-time) PCR |
| PT | proficiency testing |
| $R^2$ | coefficient of determination |
| RSD | relative standard deviation |
| SI | International System of Units |
| TF | Task force |
| *Taq* | *Thermus aquaticus* (polymerase) |
| WG | working group |

## *Abstract*

The aim of this guidance document is to facilitate harmonised flexible scope accreditation within Europe, according to ISO/IEC 17025:2005 related to quantitative testing of genetically modified organisms (GMOs) by quantitative real-time polymerase chain reaction (qPCR) for GM events authorised in the EU or which are in the authorisation process.

This document gives guidance to and is intended for laboratories that are acquiring or are holding a flexible scope of accreditation according to ISO/IEC 17025. At the same time it aims to provide information for assessors involved in the accreditation process of these laboratories.

This guidance document has been written by members of the Task Force (TF) *Flexible scope accreditation*, which has been initiated by European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (EC JRC-IRMM, Geel, BE). After an extensive commenting phase it has been approved by the European co-operation for Accreditation (EA) as an EA guidance document.

# *1 Scope of the guidance document*

The aim of this guidance document is to facilitate harmonised flexible scope accreditation within Europe, according to ISO/IEC 17025:2005 [1] related to quantitative testing of genetically modified organisms (GMOs). Considering that polymerase chain reaction (PCR) is the method of choice in the European Union (EU) for the identification and quantification of GMOs, this document refers exclusively to quantitative real-time PCR (qPCR) and GM events authorised in the EU. A validated quantification method is published by the European Union Reference Laboratory for Genetically Modified Food and Feed (EURL-GMFF) for each of the authorised events as laid down in Regulation (EC) No 1829/2003 [2]. This document is applicable to methods for GM events which were previously authorised in the EU or which are in the authorisation process, provided that the method validation has been completed and that a certified reference material (CRM) is available as requested in the low level presence (LLP) regulation for feed [3]. This guidance document does not cover screening methods, qualitative PCR methods or methods to quantify GM events not authorised in the EU.
In the future, an extension to this document might be considered dealing with those specific cases. However, some general principles illustrated here might also be applicable for the methods currently not covered.

This guidance document is intended for laboratories that are acquiring or holding a flexible scope of accreditation according to ISO/IEC 17025, and aims to provide information for assessors involved in the accreditation process of these laboratories. It therefore addresses primarily laboratory managers and assessors for ISO/IEC 17025.
This document refers to other documents, which may be reviewed and updated. As a consequence this guidance document will be updated when needed. ISO/IEC 17025 remains the authoritative document and, in case of dispute, the individual accreditation bodies will adjudicate on unresolved matters.

This guidance document has been written by the members of the Task Force (TF) *Flexible scope accreditation*, which has been initiated by European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (EC JRC-IRMM, Geel, BE) and which reported to the European Network of GMO Laboratories (ENGL). It passed an extensive commenting phase involving the ENGL and has been by the European co-operation for Accreditation (EA) in September 2013 as EA guidance document.
We welcome further constructive feedback on this *European technical guidance document for the flexible scope accreditation of laboratories quantifying GMOs*. All correspondence should be sent by email to JRC-IRMM-GMO@ec.europa.eu with the subject heading 'flexible scope document'.

## *2 Introduction*

Legislation in the EU regulates the placing on the market of food and feed consisting of, containing or produced from GMOs. They are referred to as 'GM food and feed' and require authorisation before being placed on the market in the EU. Food and feed products which contain, consist of or are produced from GMOs in a proportion higher than 0.9 per cent of the food and feed ingredient considered individually or food or feed consisting of a single ingredient, need to be labelled [2]. In this context, it needs to be noted that the labelling threshold is applicable for adventitious presence of GMOs, while GMOs added on purpose need to be labelled independent from a threshold.
Additionally feed may contain 0.1 mass per cent of a GM event which was previously authorised in the EU or for which an authorisation process is pending [3].

During the EU authorisation process, the applicant seeking authorisation for a GM event needs to ensure that a reference material for the GM event is available and that an event-specific quantification method has been successfully validated and is published by the EURL-GMFF. Successfully validated methods fulfil the minimum performance criteria laid down by the EURL-GMFF in [4]. As a consequence, (certified) reference materials[1] and validated methods are publically available to GMO testing laboratories for the GM events covered by this guidance document.

As the number of new GM events for which authorisation is granted, is increasing rapidly every year and as GMO testing laboratories are obliged to operate under ISO/IEC 17025 accreditation [5], the testing laboratories need to take up new GM events within their scope of accreditation in a timely manner. As a consequence, a flexible scope accreditation is requested by more and more GMO testing laboratories. The number of matrices on which the quantification method needs to be applied is also increasing.

[1] Authorisation according to (EC) No 1829/2003 requires the availability of a reference material. The low level legislation for feed (EC) No 619/2011 requires the availability of a certified reference material.

## *3 Flexible scope — ISO/IEC 17025, Section 1.2*

The accreditation of laboratories is based on a defined scope of accreditation which is clear and unambiguous, and provides the laboratory and other interested parties with a detailed list of the tests for which the laboratory is accredited. A precise description of the specific tests for which the laboratory is deemed competent is needed for a fixed as well as flexible scope.
A fixed scope of accreditation requires an evaluation of the laboratory's competence by the accreditation body for each new test that is added to the scope. A flexible scope allows adding a new test based on a competence evaluation carried out by the laboratory. This inclusion of a new test to the flexible scope is verified by the accreditation body *a posteriori*.

It has become desirable to establish mechanisms which permit more laboratories to extend the range of their scope on the basis that their competence related to GMO quantification by qPCR has already been evaluated.

A flexible scope for the measurement of the GM content is needed by the laboratories, allowing the quantification of GM events newly authorised in Europe without prior approval by the accreditation body. As the necessity for such flexibility is clearly established, the additional efforts to develop, implement and maintain an extended management system that a flexible scope requires can be beneficial.

A laboratory's scope of accreditation is laid down in the accreditation document and refers to one or more of the following items.

- **Product**

  The materials, in which the GM content is quantified, can be classified as being seed or food or feed, including their ground forms. Accredited laboratories are in some cases accredited only for one of these specific products.

  Note: 'Seed' has to be understood in this context as seeds suitable for agricultural purposes. Laboratories accredited for food or feed matrices only, do not test seeds but test food/feed products, while the food/feed can consist of grains (harvested material).

  Furthermore, laboratories can be explicitly accredited for GM quantification of vegetative parts from plants (e.g. potato tubers, plant leaves), while others are accredited for GM quantification in plant material in the general sense.

- **GM event**

  GM event refers to the unique DNA recombination event that took place in one plant cell, which was then used to generate transgenic plants. GM event-specific methods are targeting the unique insertion region of the DNA construct (junction between transgenic and conventional DNA sequence, further referred to as the transgene-specific DNA target).

  The concentration of the GM event is calculated as the ratio of two small (e.g. 60 to 150 bp) DNA fragments present in DNA extracted from the material tested. The relative concentration of those two fragments amplified by qPCR is determined. One of the two fragments is chosen to be specific to a particular GM event whereas the

other fragment is specific to the taxon or species (further referred to as the taxon-specific DNA target). The qPCR measurement result is expressed as ratio of those two relative DNA fragment concentrations.

Note: The measurement result can be expressed in GM mass fractions or in GM DNA copy numbers. In this context the material used for the calibration has to be taken into account.

- **Analytical procedure**

  The analytical procedure applied for the quantification of GMO is composed of a DNA extraction method, which can be based on different principles, and a DNA quantification method based on the measurement principle qPCR.

  Note: Due to the fact that the here concerned GM events are authorised in the EU, European standardised methods validated by interlaboratory comparison (ILC) organised by the EURL-GMFF and completed with the help of the ENGL are available.

  As the methods applied for GM labelling in the EU need to be event-specific [6], all methods concerned here are based on qPCR.

- **Range of measurements**

  For some accredited laboratories, the scope specifies additionally the content range of measurement results for which the accreditation is valid.

  Note: Specifying a range of measurements might be meaningful when other analytical techniques than qPCR are concerned.

## *3.1 Levels and degrees of flexibility*

The flexibility of the scope may cover three categories. For each of the below mentioned categories (Sections 3.1.1, 3.1.2 and 3.1.3) the level of flexibility can be adjusted and combined depending on the needs and degree of expertise of the accredited laboratory.

Figure 1 schematically compares a fixed accreditation scope with the flexible accreditation scope. In general, it is reasonable to grant a flexible scope to a laboratory that has proven its competence for qPCR.

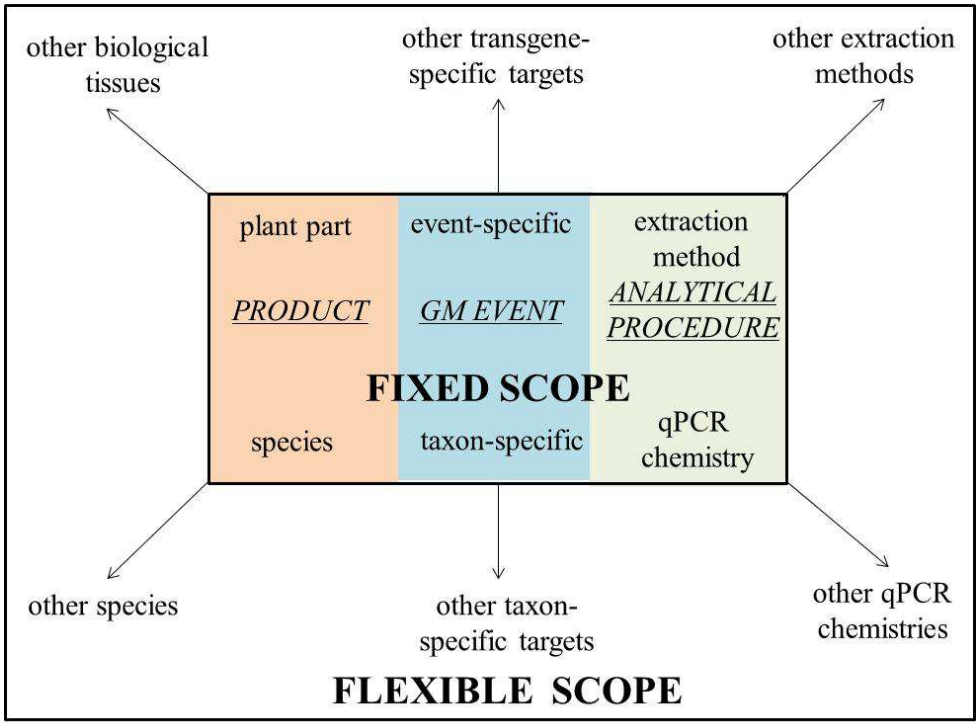


**Figure 1**: Schematic representation of a typical fixed scope for a defined GM event (e.g. MON810 and *hmg*) measured by a defined analytical procedure (e.g. CTAB and *Taq*Man chemistry) in a defined product (e.g. maize seeds). The possibilities for a flexible scope within the three categories are indicated by the arrows.

### *3.1.1 Flexibility concerning the product*

This flexibility allows for changes in the specific products tested if this can be done using the same testing techniques for the test parameters for which the laboratory is already accredited. The level of flexibility will depend on the products included in the validation of the method.

A typical fixed scope could be for MON810 maize seed, a flexible scope could be on GM seeds (in this case not limited to GM maize but extended to any other GM species) or further extended to GM plant materials (in this case not limited to GM seeds but extended to other parts of the maize plant (e.g. leaves)).

Flexibility concerning the product can be extended to products processed in different ways. For example, GM rice can be processed into different product types, such as rice flour, flaked rice grains, rice starch, roasted cereals obtained from rice or pre-cooked rice.

### *3.1.2 Flexibility concerning the GM event*

GMO quantification by qPCR using *Taq*Man chemistry validated per GM event (e.g. for the MON810, MON863 or DAS1507 maize event) could be considered to be part of a fixed scope. However, a scope defined as 'quantification of GM maize' or 'quantification of GM species' by qPCR would provide different levels of flexibility. In both cases the same measurement principle will be used (qPCR). The only difference is the DNA targeted by the primers and probes. In the first case (quantification of GM maize) only the event-specific target changes, while the taxon-specific remains limited to one species (maize). For changes

of the taxon-specific target the reader is referred to Section 3.1.3. In the second case (quantification of GM species) both targets change.

### *3.1.3 Flexibility concerning the analytical procedure*

This flexibility allows for changes in the analytical procedure of a particular method for a specific product for which the laboratory is accredited. This can concern DNA extraction methods as well as DNA quantification methods by qPCR.

For example, a typical fixed scope could be a DNA extraction using the CTAB method, a flexible scope could comprise different DNA extraction methods.

An extraction method used for another material (than the one it was verified for) may have to be adapted to meet the DNA quality criteria required for qPCR. Changes to improve the performance of the method in terms of higher yield of DNA or better PCR quality may concern the sample intake, sample preparation or clean-up procedure for a specific matrix. Different extraction methods may be needed to extract DNA from processed food or feed containing the same species.

For PCR, parameters such as PCR efficiencies and PCR linearities can be used to monitor the effects of small changes (e.g. changes in the PCR annealing temperatures, in the primer or probe concentrations, changes in the nature of the fluorescent probes and quenchers) on the performance of the testing method.

Laboratories may also decide to replace a set of PCR primer specific to a particular taxon-specific gene (e.g. the replacement of *adh*1, used as taxon-specific gene for maize with another taxon-specific gene such as *hmg*; or the replacement of a set of primers targeting different parts of the same genetic element).

In each case, the laboratory would need to demonstrate during method verification that with those changes, the criteria of acceptance of the validated method are still met.

Methods validated by the EURL-GMFF and concerned in this document have proven during validation that the minimum performance requirements (MPR) for the regulatory purpose laid down in [4] can be fulfilled. For the implementation the performance criteria required and to be achieved by the GMO testing laboratory need to be clearly described in a specific verification plan and need to fulfil the requirements laid down in [7]. It is the obligation of the laboratory to demonstrate during method verification that the method is fit for purpose in this laboratory and can be applied under a flexible scope accreditation.

## *3.2 Additional specific requirements*

In order to acquire a flexible scope accreditation, the laboratory holding a fixed scope accreditation needs to introduce a number of procedures governing the management of the flexible scope and ensuring the integrity of the introduction of further GMO quantification methods. The laboratory needs to set out clear criteria when a working instruction for a method is considered under the flexible scope.

An explicit statement needs to be produced declaring that the method is to be included in the flexible scope of accreditation, showing the timing of the inclusion and the criteria on which

this inclusion is based. The scope of accreditation needs to be updated when new methods are included according to the guidelines of the individual accreditation body. Additional requirements can be found in the document *EA requirements for the accreditation of flexible scopes* [8].

## *4 Laboratory sample preparation — ISO/IEC 17025, Section 5.4*

Some indications and general recommendations related to sample preparation are given in the International Standards ISO 21571:2005 [9] and ISO 24276:2006 [10]. Furthermore, the ENGL is working on a guideline dedicated to sample preparation.

The following aspects have to be taken into account:

- homogeneity of the laboratory sample;
- representativeness of the analytical sample and test portion with regard to the laboratory sample;
- measures to avoid cross-contamination have to be taken by the laboratory (ensuring premises are compliant, including dusting and cleaning).

Specific recommendations for those aspects can be found in the following documents:

- test portion and particle size: ISO 21571:2005, Section 5.1 [9]
- liquid samples: ISO 21571:2005, Section 5.1.2 [9]
- pasty samples: ISO 21571:2005, Section 5.1.2 [9]
- viscous samples: ISO 21571:2005, Section 5.1.2 [9]
- heterogeneous sample: ISO 21571:2005, Section 5.1.2 [9]
- premises: ISO 24276:2006, Section 5.3.2 [10]

# *5 Method verification and measurement uncertainty estimation — ISO/IEC 17025, Sections 5.4.5 and 5.4.6*

## *5.1. General considerations*

An accredited laboratory shall have a management system in place to provide objective evidence that the personnel is adequately qualified and trained to perform the analysis (ISO/IEC 17025:2005, Section 5.2 [1]). In addition, a metrology system shall ensure that the equipment used is periodically calibrated (ISO/IEC 17025:2005, Section 5.5 [1]). When a method validated by ILC is used by an accredited laboratory, the laboratory must, prior to its use, ensure that the chosen method shows performance characteristics as good as or better than those assessed in the ILC. This verification process must be documented and recorded in the quality system [7]. If the method performance characteristics investigated in the ILC cannot be met, then the method performance needs to be improved by the laboratory.

The laboratory shall establish the criteria for the acceptance of the verification results. These criteria need to be set in such a way that successful verification confirms that the method is suitable for the intended purpose. The laboratory must record the procedure used, the results obtained and a statement on whether the method is fit for the intended purpose, e.g.:

- design and planning of the verification
- description of the method applied
- acceptance criteria and performance requirements, as decided by the laboratory
- test records of the verification measurements
- documentation of the conclusion

## *5.2 Method verification*

Method verification concerns in the given context methods that have already been validated by third parties.

The document *Definition of minimum performance requirements for analytical methods of GMO testing* [4] used by the EURL-GMFF for the assessment of GMO detection methods submitted by applicants within the frame of the Regulation (EC) No 1829/2003 [2] should be used as a basis for assessing the performance of a method. Additionally, a working group (WG) of the ENGL has published guidelines for the implementation/adaptation of such validated methods in control laboratories of the EU. The document entitled *Verification of analytical methods for GMO testing when implementing interlaboratory validated methods* [7] gives guidance on how to verify that a validated method performs sufficiently for control purposes in a given laboratory. The guidelines are intended for laboratories accredited with a fixed or flexible scope under ISO/IEC 17025. In the following paragraphs, only the general information contained in the guidelines published by the ENGL WG is summarised.

ISO/IEC 17025 accreditation states that '*The laboratory shall confirm that it can properly operate standard methods before introducing the tests or calibrations'*. If the standard method changes, the confirmation shall be repeated (ISO/IEC 17025:2005, Section 5.4.2 [1]). In GMO detection laboratories, the event-specific method provided during the application for authorisation is used for GMO quantification. This method has been validated by the EURL-

GMFF in collaboration with the ENGL and is available via the website of the EURL-GMFF and the *Compendium of reference methods for GMO analysis* [11]. This compendium contains qPCR-based GMO detection methods that have been validated through ILCs according to ISO 5725-2:1994 [12] and/or the IUPAC protocol [13]. Before applying such a validated method for GMO testing, the GMO testing laboratory needs to confirm that it is able to properly operate the method. This confirmation is done during method verification.

### *5.2.1 Parameters for method verification*

The following sections describe the parameters to be studied for the verification of validated methods for the quantification of GMOs. During the verification process, a laboratory should ensure compliance with the requirements described in the following documentary standards ISO 21569:2005 [14], ISO 21570:2005 [15], ISO 21571:2005 [9] and ISO 24276:2006 [10].

As a matter of principle, a method should be implemented as validated in the ILC, without introducing modifications. If single elements like for example the brand of a ready-to-use reaction mix or *Taq* polymerase, the PCR reaction volume, the primer and probe concentrations, and/or PCR cycling parameters are modified, it needs to be ensured that the MPR laid down in [4] are still fulfilled. The proof that a change of a general PCR condition does not invalidate the validation data may be carried out on a limited number of PCR methods selected by the laboratory. However, if the laboratory decided to verify the changes on a limited number of PCR method (and not on all), it is the responsibility of the laboratory to ensure that the selection of methods is meaningful. It is for instances recommendable to include methods targeting various species as well as methods that are less robust.

At the time of the method verification CRMs are available to the laboratories. Therefore, the verification process is preferably conducted on CRMs. If no CRM is available at the proper concentration levels, additional steps have to be considered (Section 7).

#### *5.2.1.1 Sample preparation*

An essential step for obtaining reliable GM measurement result is the sample preparation or grinding of the samples to be analysed. Grinding helps to ensure the homogeneity of the tested batch and thus the representativeness of the test portion analysed. Moreover, it can facilitate the DNA extractability.

In general, more DNA can be extracted per mass unit from a sample with a smaller average particle size. It is therefore important to determine the minimum sample size and to consider the maximum particle size required to reach a specific limit of detection (LOD) using a given sample intake. For example, seed/grain samples contaminated at the level of the LOD of the method should be ground and tested each independently in six test portions (analogue to the checking of the DNA extraction in six test portions [7]). If all test portions test positive, the grinding conditions are suitable and the test portion can be considered as sufficiently homogeneous.

If the test portions give different results, the grinding conditions do not lead to sufficiently homogeneous material and need to be improved.

Alternatively the particle size can be measured using a particle size analyser or a sieving test and the average particle number in the test portion estimated. If each test portion contains at

least 3000 particles, an LOD of 0.1 (m/m) % can be assured assuming a homogeneous Poisson distribution. If there are reasons to assume that the contamination in the samples are likely to be heterogeneous, it is better to ensure that the test portion contains at least 10000 particles which would allow for a heterogeneous Poisson distribution.

### *5.2.1.2 DNA extraction*

The DNA extraction method should provide DNA of suitable quality and quantity for subsequent analysis. DNA extraction and the selection of a method are crucial, as the quality and quantity of DNA extracted can significantly affect the final result. Two cases can be considered depending on whether or not the applied DNA extraction method has been previously and appropriately validated.

If the DNA extraction method has been previously validated (either by the EURL-GMFF or another organisation) for the purpose in question (similar matrix, etc.), the laboratory has to check whether the method delivers also in this specific laboratory DNA extracts suitable for PCR and meets the criteria set out in the document *Verification of analytical methods for GMO testing when implementing interlaboratory validated methods* [7].

Procedure: DNA extraction is carried out at least twice (three times recommended) each time on two test portions, if possible on different days and by different operators.

Acceptance criteria: The DNA extractions should meet the acceptance criteria for DNA concentration and quality (e.g. by checking amplification efficiency and testing for the absence of inhibitors by qPCR) [7].

Note: DNA extraction methods applied to one matrix may not be suitable for other matrices, while the intention is to apply the extraction method on different matrices. For the verification of a DNA extraction method the tested matrix does not necessarily have to contain GMOs. However, it needs to be tested on the GM event if the event modifies the composition of the material in a way that might have an impact on the DNA extractability (e.g. starch-modified potatoes).

If the DNA extraction method has not been previously validated for the purpose, the laboratory has to check whether the method delivers DNA extracts suitable for PCR. This is usually done in a single laboratory validation.

Procedure: The DNA extraction is carried out at least five times on the same sample, if possible on different days and by different operators.

Acceptance criteria: The DNA extract should meet the acceptance criteria for DNA concentration and quality (e.g. by checking amplification efficiency and testing for the absence of inhibitors by qPCR) which have been reasonably set by the laboratory, e.g. by using acceptance criteria from other internationally validated extraction methods for the same plant species, or for the same type of food or feed product [7].

At this stage the laboratory has validated the DNA extraction method for the matrix used during the validation. If the method is intended to be applied for other matrices the laboratory needs to verify DNA extraction methods for the other matrices concerned or even broaden

the scope of the validation by using the most relevant matrices for which it requests a flexible scope accreditation. For food and feed products it is recommended to use at least three different product types should be tested per species (e.g. rice flour, flaked rice grains, rice starch). If the laboratory only deals with one specific product type, the verification can be based on this one product type only. The matrices which were tested and the conclusions drawn for the applicability of the validated method need to be clearly stated by the laboratory.

### *5.2.1.3 DNA concentration*

Procedure: After applying the DNA extraction method on routine samples, the concentration of DNA in the extracts should be measured.

Acceptance criterion: In the verification process, when a DNA extraction method is applied to the same matrix as in the validation study, the amount of DNA extracted should be at least equal to the results obtained in that study. The method should provide DNA in an appropriate amount for the intended analysis, at least enough to meet the desired LOD and LOQ and the required representativeness.
Note: The DNA yield of an extraction method depends strongly on the material used. Even the same type of matrix can lead to differences (e.g. fresh maize grains *versus* old grains). Furthermore, the composition of the samples can influence the DNA amount measured and the amount does not necessarily give an indication about the PCR amplifiability of the DNA. However, comparison with similar samples and the measured DNA concentration allows to draw conclusions on the suitability for PCR. If a DNA extraction method does not give an appropriate yield for the intended analysis on a particular matrix, the LOD will be affected [7] and the impact needs to be evaluated.
For the impact of the particle size check Section 5.2.1.1.

### *5.2.1.4 Absence of inhibitors*

Inhibition of a PCR reaction may depend on the sample from which the DNA is extracted and on the DNA extraction method applied. Therefore an inhibition check needs to be carried out unless it can be proven (during method verification) that a certain sample/method combination does not lead to inhibition.

Procedure: Each DNA extraction replicate obtained from the sample is diluted as done during routine analysis (further referred to as working dilution). From this working dilution, a dilution series of, for example, four concentrations are analysed by qPCR (at least two PCR replicates per dilution). The measurement results are used to obtain a calibration curve.

The preferred PCR assay for the inhibition test is targeting the taxon-specific DNA. The total DNA amount in the working dilution should be at least the same as the total DNA amount intended to be used in the verification process and in the later routine analysis (e.g. the DNA amount indicated in the PCR protocol for the taxon-specific measurement).

Acceptance criterion: From a four-fold calibration curve the average difference (ΔCq) between the measured Cq value of the dilution and the calculated Cq value of the further dilution (calculated from extrapolation of the Cq values of the following dilutions) should be

< 0.5 [measured Cq – extrapolated Cq < 0.5] and the slope of the calibration curve should lie between –3.1 and –3.6 [7].

If the extracted DNA solution contains inhibitors, the DNA has to be further purified or diluted to the level where no inhibition of the PCR reaction is observed before it is used for qPCR.

### *5.2.1.5 Specificity*

Specificity has already been investigated in the context of method validation; the specificity does therefore not need to be experimentally investigated during verification. In case of doubt the specificity of the method can be verified experimentally using e.g. CRMs. Additionally it can be helpful to investigate the absence of high sequence similarity *in silico* between the amplicons and a database using dedicated algorithms for comparing primary DNA sequence information (e.g. Basic Local Alignment Search Tool). However, it has to be born in mind that the transgenic sequence cannot be covered by the latter approach.

### *5.2.1.6 Linear range, $R^2$ coefficient, and amplification efficiency*

Procedure: Linear range, coefficient of determination ($R^2$), and amplification efficiency are verified simultaneously from calibration curves when testing other parameters, such as trueness and precision. The mean values of at least two calibration curves should be taken.

Acceptance criterion for linear range: The linear range must cover the values corresponding to the expected use which is typically the legal labelling threshold of 0.9 % for authorised events [2] or 0.1 (m/m) % for LLP events [3]. The linear range should be expressed in either GMO mass fractions or copy number ratios, while stating clearly which measurement unit has been used. Mass fractions can be expressed as g/kg or as a percentage (g/100 g). Preferably copy number ratios are also expressed as a percentage.

Note: The linear range mentioned above is called the ‘dynamic range’ and is defined as the range of concentrations for which the method has with an acceptable level of trueness and precision a linear relation between the logarithm of the concentrations and the Cq values. The decadic logarithm of the concentration is plotted against and the Cq values to generate a calibration curve.

Acceptance criterion for $R^2$ coefficient: The recommended average value of $R^2$ shall be $\geq 0.98$.

Acceptance criterion for amplification efficiency: For quantitative methods, the recommended average value of the slope of the calibration curve shall be in the range of –3.1 and –3.6 [7].

### *5.2.1.7 Trueness and bias*

Trueness is the qualitative expression of the closeness of agreement between a measured a reference (usually the certified value of a certified reference material). Bias is the quantitative expression of trueness.

Procedure: The trueness should be determined at a level close to the level of decision (typically the legal labelling threshold of 0.9 % for authorised events [2] or 0.1 (m/m) % for LLP events [3]). Preferably, as outlined in [7] trueness should, if possible be verified at two level, e.g. the level of decision and the LOQ. Any bias detected needs to be eliminated or corrected (Section 5.2.2.3) in order not to hamper the trueness of the obtained measurement results.

Acceptance criterion: If no significant difference is observed between the measurement results obtained on the CRM and the certified value, considering both uncertainties, the trueness of the method is confirmed [16].

If no CRM is available with the required GM mass fraction additional steps need to be taken (Section 7.2). Alternatively the outcome achieved by a laboratory within a PT can be used to verify if the reported value is in agreement with the consensus value. Acceptance criterion: A z-score and a zeta-score between 2 and -2 can be considered as satisfactory.

Note: The interpretation of z-scores alone needs to be handled with care as the consensus value may differ from the true value. For the evaluation of the PT outcome the expected GM level (e.g. from the spiking of the PT sample) should be taken into account.

### *5.2.1.8 Relative repeatability standard deviation*

Procedure: The relative repeatability standard deviation ($RSD_r$) can be determined in a way similar to that described under trueness (Section 5.2.1.7). It is calculated from PCR replicates run under repeatability conditions. Repeatability should be investigated for different GM concentrations using the same procedure and instrument under the same conditions within a short period of time.

The testing conditions (reaction volume, PCR machine, number of extractions and number of PCR replicates, etc.) should be the same as those during routine analysis of samples. Results from at least 16 PCR replicates should be obtained. Examples for possible test designs can be found elsewhere [7].
Acceptance criterion: $RSD_r$ should be $\leq$ 25 % (calculated on the measured GMO content), over the dynamic range of the method.

### *5.2.1.9 Limit of detection*

Procedure: A positive control material of low GM concentration can be measured in 10 PCR replicates, and if all replicates are positive, this infers that the LOD is with a 95 % confidence level below or equal to the positive control material concentration. One possible way of calculating the LOD is given in [17], an alternative is outlined in [7].

Acceptance criterion: Whenever validation data are available the LOD should be in line with those data [7].

Note: During method verification the laboratory established the LOD with a certain confidence level, usually a LOD with 95 % confidence interval is calculated. This LOD and its confidence level are based on a number of measurements. During the routine application of qPCR less than 10 (usually 2 to 3 measurements) are performed. As qPCR ideally detects

each single amplifiable molecule in the reaction tube, a discrepancy can sometimes be observed between the results obtained during LOD establishment and during routine analysis.

The reason for this discrepancy is that 2 to 3 positive measurement results may be observed even at this confidence level of 30 %.

### *5.2.1.10 Limit of quantification*

Procedure: A positive control material of, for example, 1 g/kg (0.1 (m/m) %) can be analysed in 10 PCR replicates of the GM target and 10 replicates of the taxon-specific gene target. The $RSD_r$ at the LOQ should be below 25 % (calculated on the measured GMO content). To establish the true LOQ, it would be necessary to make dilutions to a lower GM content (for further guidance see [7]). Besides the $RSD_r$ established at the LOQ, the trueness of the measurement should be verified during method verification (Section 5.2.1.7).

Acceptance criterion: Whenever validation data are available, the LOQ should be in line with (or better than) those data [7].

Note: By establishing the LOQ on 10 PCR replicates derived from the same DNA extract, the potential effect of the DNA extraction method is not covered.

To ensure that the DNA concentration has not been overestimated, a test could be performed. A nominal dilution of 0.1 copy per PCR reaction is tested in six replicates. No more than one out of the six replicates should create a positive measurement signal [7].

## *5.2.2 Measurement uncertainty estimation*

Measurement uncertainty (MU) is estimated using data obtained on samples within a given laboratory, establishing the intermediate measurement precision. There are different possibilities to establish the intermediate measurement precision, one option is to use the data generated during method verification [7]. Another option is using data obtained on laboratory samples (such as internal quality control or routine sample data). The accessibility of samples might influence which approach is to be used.
GMO laboratories may also use information derived from the following procedures to aid their estimation of the uncertainty of measurement results:

- the ISO/IEC Guide 98-3: *Guide to the expression of uncertainty in measurement* [18]
- the EURACHEM/CITAC Guide CG4: Q*uantifying uncertainty in analytical measurement* [19].

The possibility to use data obtained on routine samples has been outlined, together with other options, in the *Guidance Document on Measurement Uncertainty for GMO Testing Laboratories* [17]. In this document worked-out examples are provided. The general approach is to estimate the intermediate measurement precision using the repeated independent analyses of a range of real samples and add the uncertainty connected to the bias control. This general approach is valid as long as no significant measurement bias has been found during the bias control and is based on the Nordtest report [20] and outlined in Sections 5.2.2.1 to 5.2.2.6 of this document.

Note: MU estimation is independent from the unit of measurement (being either mass fractions (m/m) or copy number ratios) and is carried out in the same way for either unit of measurement.

MU is a single parameter that describes the quality of measurement and is linked to the individual measurement performed and each laboratory has to evaluate the specific MU for a measurement result obtained under defined conditions.

MU is ideally estimated using routine samples as this ensures that the characteristics of the samples intended to be analysed are taken into account. However, enough routine samples are not always available or accessible to the routine laboratories and alternatives might need to be considered (Section 5.2.2.1, Note).
MU should take into account all effects on a measurement process. If sampling is outside the control of the testing laboratory, and it is considered as a meaningful component in the MU budget, it should explicitly be stated that the uncertainty budget does not cover the sampling uncertainty.

A control laboratory shall always estimate [1] and should report the MU associated with their analytical results.

### *5.2.2.1 Measurement uncertainty estimation using intermediate precision*

The general approach is to estimate the intermediate precision and to determine it by repeated independent analysis of samples in analytical runs that represent the long-term variation of analytical components within the laboratory, e.g. different operators, stock solutions, new batches of critical reagents, recalibrations of equipment, etc. Also, samples should represent the different matrices and concentrations to which the estimates of MU will be applied. In particular, samples with a GMO content close to the thresholds against which results will be compared should be included (which is typically the legal labelling threshold of 0.9 % for authorised events [2] or 0.1 (m/m) % for LLP events [3]). MU estimates should be updated or at least verified as new results become available.

Repeated independent results produced on at least 15 samples should be used ($N \geq 15$). In order to maximise the matrices and concentrations studied it is recommended that the smallest replication e.g. two independent measurements ($n = 2$) per sample (two extractions from the same sample), is applied to the largest number of samples possible.

For the following approach the observation that the measurement uncertainty is composed of a constant part ($u_0$) which is independent of the GM content measured and a relative part ($u_{pro}$) which is proportional to the GM content measured is used. Measurement uncertainty is therefore dominated by $u_0$ when lower GM contents are measured and by $u_{pro}$ when higher GM contents are measured. Both aspects are taken into consideration in the following approach.

The standard deviation ($s$) is calculated twice for two different situations. Once using the six results with the lowest mean GM content ($N = 6$, $n = 2$) and once using the remaining samples with higher mean GM content ($N \geq 9$, $n = 2$). The standard deviation derived from results with low GM content gives the constant part of the measurement uncertainty ($u_0$) and needs to be combined with the standard deviation derived from samples with higher GM content ($u_{pro}$) and the uncertainty related to the bias ($u_{bias}$), together representing the

proportional part of the measurement uncertainty ($u_{pro,bias}$). In this way the repeatability component will be included twice, but is normally still small in comparison to the between-day variation. For the same reasoning the resulting standard deviation ($s$) is directly used (Equation 4 and 5) to estimate MU as $u = s$ and not divided by the square root of the number of measurements ($n$). This approach is valid if the repeatability of the method is negligible compared to the day-to-day variation. For further details see [20].

Note: The availability of samples is often the limiting factor in the above described approach. The laboratory may be forced to estimate the MU on fewer samples and/or different sample matrices (in the extreme case only using a reference material). In such cases the laboratory should consider to add an uncertainty component for the parts which can (currently) not be investigated due to the lack of samples representative for routine analysis. Such an additional uncertainty component can for instance be estimated on the basis of observations made with other species and/or matrices and is referred to as reconciliation procedure [19].

It is in all cases recommendable that the laboratory verifies that the estimated MU covers the observed scatter of measurement results observed during routine measurements. If this is not the case, this is an indication that the MU has been underestimated and needs to be reconsidered (Section 5.2.2.3, Equation 7).

### *5.2.2.2 Intermediate precision*

The mean ($\overline{c_i}$) of two independent analytical results is calculated as (Equation 1):

$$\overline{c_{\mathrm{i}}} = \frac{c_{\mathrm{i},1} + c_{\mathrm{i},2}}{2}$$

| | |
|---|---|
| $\overline{c_{\mathrm{i}}}$ | mean of two analytical results |
| $c_{\mathrm{i},1}$ | result of first analysis of sample *i* |
| $c_{\mathrm{i},2}$ | result of second analysis of sample *i* |

The absolute difference ($d_i$) between the first and the second analysis is calculated as (Equation 2):

$$d_i = \left|c_{\mathrm{i},1} - c_{\mathrm{i},2}\right|$$

| | |
|---|---|
| $d_{\mathrm{i}}$ | absolute difference between two analytical results |
| $c_{\mathrm{i},1}$ | result of first analysis of sample *i* |
| $c_{\mathrm{i},2}$ | result of second analysis of sample *i* |

The relative difference between analyses ($d_{\mathrm{i,rel}}$) is calculated in per cent as (Equation 3):

$$d_{\mathrm{i,rel}} = \frac{d_{\mathrm{i}}}{\overline{c_{\mathrm{i}}}} \cdot 100$$

| | |
|---|---|
| $d_{\mathrm{i,rel}}$ | relative difference |
| $d_{\mathrm{i}}$ | absolute difference between two analytical results |
| $\overline{c_{\mathrm{i}}}$ | mean of two analytical results |

Given a set of differences and relative differences calculated from the analysis of a number of samples ($N \geq 15$, $n = 2$) the mean difference ($\bar{d}$) and mean relative difference ($\overline{d_{i,rel}}$) can be calculated. Afterwards the data derived from the six results with the lowest mean GM content ($N = 6$, $n = 2$) are used to calculate $s_0$ and the remaining samples with higher mean GM content ($N \geq 9$, $n = 2$) are used to calculate $s_{pro}$.
In the case of two independent measurements results ($n = 2$), $s_0$ is estimated via (Equation 4):

$$s_0 = \frac{\bar{d}}{d_2} = \frac{\bar{d}}{1.13} = u_0$$

| | |
|---|---|
| $s_0$ | standard deviation associated with samples with a lower GM content |
| $\bar{d}$ | mean difference |
| $d_2$ | 1.13 (rounded constant depending on the number of independent measurements ($n$) [20]) |
| $u_0$ | measurement uncertainty associated with samples with lower GM content |

The standard deviation associated with samples with higher GM content ($s_{pro}$) is estimated as a relative parameter ($s_{pro,rel}$) and given by (Equation 5):

$$s_{pro,rel} = \frac{\overline{d_{i,rel}}}{d_2} = \frac{\overline{d_{i,rel}}}{1.13} = u_{pro,rel}$$

| | |
|---|---|
| $s_{pro,rel}$ | relative standard deviation associated with samples with a higher GM content |
| $\overline{d_{i,rel}}$ | mean relative difference |
| $d_2$ | 1.13 (rounded constant depending on the number of independent measurements [20]) |

### *5.2.2.3 Trueness control*

A CRM with its certified value and uncertainty should be used for the trueness control. After the measurement of a CRM the bias can be quantified, for this the absolute difference between the mean measured value and the certified value can be calculated as (Equation 6):

$$d_m = |c_m - c_{CRM}|$$

| | |
|---|---|
| $d_m$ | difference between mean measurement result and certified value |
| $c_m$ | mean measurement result obtained for the CRM |
| $c_{CRM}$ | certified value of the CRM |

The uncertainty of $d_m$ is calculated from the uncertainty of the certified value and the uncertainty of the measurement result.

The uncertainty of the measurement result ($u_m$) can be estimated by dividing the standard deviation by the square root of the number of measurements carried out (Equation 7):

$$u_{\mathrm{m}} = \frac{s_{\mathrm{m}}}{\sqrt{n}}$$

| | |
|---|---|
| $u_{\mathrm{m}}$ | standard uncertainty of the measurement result |
| $s_{\mathrm{m}}$ | standard deviation of the measurement result |
| $n$ | number of independent measurement results |

The expanded uncertainties $U_{\mathrm{CRM}}$ of each certified value are given on the CRM certificate. The standard uncertainty, $u_{\mathrm{CRM}}$, of the certified value is obtained by dividing the stated expanded uncertainty by the coverage factor given on the certificate.

The combined standard uncertainty ($u_{\mathrm{c}}$) of measurement result and certified value (uncertainty of $d_{\mathrm{m}}$) is calculated according to (Equation 8):

$$u_{\mathrm{c}} = \sqrt{{u_{\mathrm{m}}}^2 + {u_{\mathrm{CRM}}}^2}$$

| | |
|---|---|
| $u_{\mathrm{c}}$ | combined standard uncertainty of the measurement result and certified value |
| $u_m$ | standard uncertainty of the measurement result |
| $u_{\mathrm{CRM}}$ | standard uncertainty of the certified value |

Note: In case of asymmetric uncertainties of the certified value, the uncertainty concerned has to be taken. For example, if the measured value is above the certified value the 'plus' uncertainty of the certified value has to be taken.

The expanded uncertainty $U$, corresponding to a confidence level of approximately 95 %, is obtained by multiplication of $u_c$ by a coverage factor (Equation 9):

$$U = k \cdot u_{\mathrm{c}}$$

| | |
|---|---|
| $U$ | expanded uncertainty of difference between result and certified value |
| $k$ | coverage factor |
| $u_{\mathrm{c}}$ | combined standard uncertainty of the measurement result and certified value |

Note: For most purposes it is recommended that a coverage factor of $k$ = 2 is used as the statistical observations have a degree of freedom of at least 6 [19].

If $d_{\mathrm{m}}$ *(*the absolute difference between the mean measured value and the certified value) is smaller or less than $U$ (the expanded uncertainty of the difference between results and certified value), then there is no significant difference between the measurement result and the certified value, meaning that the method does not have a bias. In case a bias is found, the cause has to be investigated and preferably eliminated. Approaches to calculate a bias can be found in the *Guide to the expression of uncertainty in measurements* (GUM) [19], but have to be considered carefully as a bias may be a constant or may be proportional to the measured GM concentration.

In the case that no CRM with the required concentration level is available for bias control, CRMs certified for their GM purity can be used. The standard uncertainty of a sample

produced by the laboratory has to be estimated using also the purity data of the CRMs used (Section 7.2.2).

### *5.2.2.4 Estimation of the uncertainty component associated with bias*

The relative standard uncertainty associated with the bias ($u_{\text{bias,rel}}$) is given by (Equation 10):

$$u_{\text{bias,rel}} = \sqrt{\left(\frac{s_{\text{CRM,rel}}}{\sqrt{n}}\right)^2 + \left(\frac{u_{\text{CRM}}}{c_{\text{CRM}}} \cdot 100\right)^2}$$

| | |
|---|---|
| $u_{\text{bias,rel}}$ | relative uncertainty related to the bias |
| $s_{\text{CRM,rel}}$ | relative standard deviation associated with the CRM measurement |
| $n$ | number of measurements |
| $u_{\text{CRM}}$ | standard uncertainty associated with the certified value of the CRM |
| $c_{\text{CRM}}$ | certified value of the CRM |

Note: In case of a bias the experimental set-up should be changed until no bias is found. If the bias cannot be eliminated the approach described here, of only adding the uncertainty related to the trueness control and the bias quantification ($u_{\text{bias,rel}}$), is not sufficient. In case that the bias is not eliminated, which is generally not recommended, the bias needs to be added additionally to the uncertainty estimation. For further information see [19].

### *5.2.2.5 Calculation of the proportional part of the standard uncertainty*

The proportional part of the standard uncertainty ($u_{\text{pro,rel}}$) is combined with the relative uncertainty associated with bias ($u_{\text{bias,rel}}$) using (Equation 11):

$$u_{\text{pro,bias,rel}} = \sqrt{{u_{\text{pro,rel}}}^2 + {u_{\text{bias,rel}}}^2}$$

| | |
|---|---|
| $u_{\text{pro,bias, rel}}$ | relative standard uncertainty associated with bias and samples measured with a higher GMO content |
| $u_{\text{pro,rel}}$ | relative standard uncertainty associated with samples with a higher GMO content |
| $u_{\text{bias,rel}}$ | relative standard uncertainty associated with bias |

Note: The individual standard uncertainties need to have the format of a standard deviation in order to allow summing up. Independent uncertainties can be combined by taking the square root of the sum of the individual squares [19]. $u_{\text{pro,rel}}$ and $u_{\text{bias,rel}}$ are not completely independent from each other as all measurements are influenced by the intermediate precision of the measurements. The effect of taking this twice into account for the uncertainty estimation is considered to be negligible.

### *5.2.2.6 Evaluation of measurement uncertainty*

The uncertainty contributions are assumed to be composed of a constant contribution and a contribution proportional to the measured content *c*. Both can be estimated from intermediate precision data obtained on within-laboratory samples. For the standard uncertainty, $u_0$ and $u_{\text{pro,bias, rel}}$ (Equation 4 and 11) are combined. The standard uncertainty *u* associated with a measurement result *c* is given by (Equation 12):

$$u = \sqrt{{u_0}^2 + \frac{\left(c \cdot u_{\text{pro,bias,rel}}\right)^2}{100}}$$

| | |
|---|---|
| $u_0$ | measurement uncertainty associated with samples with lower GM content |
| $c$ | measurement result |
| $u_{\text{pro,bias, rel}}$ | relative standard uncertainty associated with bias and samples measured with a higher GMO content |

Note: It has to be stressed that equation 12 is valid under the assumption that $u_0$ is constant and $u_{\text{pro,bias, rel}}$ is proportional to the GM content *c*. This assumption should be checked using in-house validation or verification data.
It can occur that $u_0$ is so small that it can be neglected.

Regulations (EC) No 1829/2003 [2] and (EC) No 1830/2003 [21] set a labelling threshold for the total authorised GMO presence on an ingredient basis. As such, the GMO contents for various events of one ingredient must be added together and the uncertainties associated with each individual GMO measurement combined. The MU of various methods can be combined by adding the squares and taking the square root of the sum (Equation 13):

$$u_c = \sqrt{\sum_{i=1,n} {u_{\text{meth,i}}}^2}$$

| | |
|---|---|
| $u_c$ | combined standard uncertainty associated with the measurement result for one ingredient |
| $n$ | number of methods applied |
| $u_{\text{meth,i}}$ | absolute standard uncertainty of individual method |

The expanded uncertainty *U* (giving a confidence level of approximately 95 %) is given by (Equation 14):

$$U = 2 \cdot u$$

| | |
|---|---|
| $U$ | expanded standard uncertainty |
| $u$ | standard uncertainty (in case of various events $u_c$) |

## *6 Measurement unit for GMO quantities — ISO/IEC 17025, Section 5.6.2.2.1*

The measurement signals of qPCR are Cq (quantification cycles), which correspond to the number of amplification cycles a DNA extract has to undergo in order to pass a set fluorescence detection threshold. The Cq value has an inverse correlation with the number of amplifiable DNA targets in the extract. But there is no simple and theoretically predictable correlation between the number of initial DNA targets and the Cq measured by qPCR. As various reaction conditions of qPCR and potential matrix effects influence the measured Cq, knowledge about the genetic composition of the plant material does not allow to predict the measurement results. In order to translate the Cq obtained for DNA extracted from an unknown sample into a measurement result, calibration of the qPCR signals is required. The calibration determines whether the measurement result is expressed in GM mass fractions or in GM-DNA copy numbers relative to target taxon-specific DNA copy numbers calculated in terms of haploid genomes.

Measurement results of GM food and feed samples can be expressed as a percentage and would be either mass fractions or GM DNA in relation to total species DNA. In both cases the measured GM content is expressed relative (either relative to the mass of the species or relative to the taxon-specific gene content).

The intended calibration approaches described in the following are meant to be used together with the EURL-GMFF validated qPCR methods.

### *6.1 Calibration of qPCR measurements for results expressed in mass fractions*

For all GM events authorised in the European Union, CRMs are available which are either certified for a GM mass fraction or for their mass-related purity (nominal 0 and 100 % GMO CRMs).

#### *6.1.1 Calibration with CRMs providing values for various mass fractions*

CRMs certified for different GM mass fraction are used to extract DNA and to set up two calibration curves: one for the transgene and one for the taxon-specific gene. Each mass fraction CRM leads in this case to a point on each calibration curve. Using the CRMs in this way ensures that dilution does not eliminate possible matrix effects of food and feed samples.

The other approach often applied, namely to extract DNA from the CRM containing the highest concentration of the GM event and to dilute the extracted DNA to set up the two calibration curves, requires a further quality control to check for possible matrix effects (Section 7.4).

It is important to set up both calibration curves from the same DNA extract and individual extracts need to be pooled beforehand. By ensuring this, possible mistakes related to DNA quantity measurements are equalled out (e.g. an overestimated DNA quantity would be equally overestimated for the transgene and taxon-specific calibration curves).

Using the certified value of the CRM and taking the dilution factors including their uncertainties into account, the transgene calibration curve allows conversion of the measured Cq for the transgene in an unknown sample into a GM mass and the taxon-specific gene calibration curve allows conversion of the measured Cq for the taxon-specific in an unknown sample into the total species mass. The ratio of both gives the GM mass fraction.

Note: In both cases of setting up a calibration curve, a minimum of 5 calibration points should be used per calibration curve [7].

### *6.1.2 Calibration in mass fractions with pure GM CRMs*

DNA is extracted from the CRM containing pure GM material and is diluted to set up the two calibration curves: one for the transgene and one for the taxon-specific gene. Afterwards the ratio of both is obtained as described in Section 6.1.1.
A further quality control check for potential matrix effects is required (Section 7.2.1 or 7.2.2).

## *6.2 Calibration of qPCR measurement results expressed in GM DNA copy number ratios*

For a few GM events authorised in the EU, CRMs for calibration of measurements expressed in haploid genome ratios are available.

Note: With respect to many plants, which are polyploid in the mitotic phase (sporophyte), the haploid genome would still represent more than one genome equivalent in the meiotic phase (gametophyte). Thus, the term 'haploid' might be misleading and should be understood in this context as 'holoploid' (for further information see [22]).

### *6.2.1 Calibration in haploid genome ratios with available CRMs*

A plasmid CRM containing both the transgenic and the taxon-specific DNA fragments, certified for its DNA sequence and suitable for calibration is used for setting up the two required calibration curves. The various calibration points are achieved by dilution of the same plasmid solution. More details can be found in [23].
Note: In both cases of setting up a calibration curve, a minimum of 5 calibration points should be used per calibration curve [7].

### *6.2.2 Calibration in haploid genome ratios in the absence of CRMs*

In the absence of a CRM for calibration, a plasmid containing the two targets (transgene and taxon-specific gene) in a known ratio can be used. Care has to be taken that the purity of the plasmid can be guaranteed (no contamination e.g. with plasmids containing a different number or ratio of the two targets) and that the plasmid is suitable for PCR amplification (identical efficiency of the targets during amplification). Information about the suitability check for calibration can be found in [24].

## *6.3 Additional notes*

(a) Calibration processes are linked to an uncertainty. In general, the uncertainty of the calibration process has to be added. To cover the reproducibility of the calibration, it is recommendable to carry out several calibrations during the method application or verification. Use of these values to estimate the MU linked to the GMO quantification measurements ensures that the uncertainty covers the reproducibility of the calibration curve.
In case of a mass fraction certified CRM the uncertainty of the calibrant is given by the certified value and its expanded uncertainty. For the certified plasmid calibrants the uncertainty has been found to be negligible. However, even for the mass fraction CRMs the uncertainty is often less than one third of the uncertainty arising from the reproducibility of the qPCR method and can therefore, using the GUM approach [19], be neglected. A careful evaluation of the uncertainty linked to the calibration process is strongly recommended. In particular the non-equivalence of plasmid and genomic DNA in the PCR process has to be considered.

(b) The conversion from measurement results expressed in mass fractions into measurement results expressed in haploid genome copy number ratios is connected with a huge uncertainty, which leads often to meaningless results as discrimination from zero is not possible anymore, for details see [25].

(c) Also the mass fraction based reference system is artificial, as qPCR quantifies DNA targets. As these DNA targets are not necessarily equally present (and accessible) in different samples of the same mass with the same GM event or species identity, the CRM and the qPCR methods set the reference system and therefore should be specified when reporting the measurement results. One should be aware that also the use of several CRMs with different mass fractions for the calibration curve compared to one CRM (dilution of the extracted DNA) influences the reference system. In the first case the reference system is based on the two varieties used as GM and non-GM components in the CRMs: in the second case the reference system is based on the variety used as GM material.

(d) The application of the ΔCq method is generally not recommended. The ΔCq method uses one calibration curve for the ratio of transgenic and taxon-specific DNA fragments and depends on identical efficiencies of the PCR amplification of transgene and taxon-specific target genes, which is often not realised. Before applying a ΔCq method it needs to be verified that the PCR amplification efficiencies on both, the transgenic target and the taxon-specific target are not significantly different from each other. This is checked during method validation and verification. In this case DNA is extracted from various CRMs and used to establish the required calibration curve. The validation which would be required after a transformation of a validated ΔCq method into a qPCR method using two standards curves is not covered by this document. The related minimum performance requirements which would need to be met during the validation of the qPCR method are outlined in [4].

## *7 Selection and use of reference material — ISO/IEC 17025, Section 5.6.3*

CRMs with certified values traceable to SI Units of measurement shall be used when available [1]. The quality control with a CRM verifies if a correct measurement procedure (including calibration) has been carried out and if the measurement results obtained on routine samples can be trusted. Only CRMs certified for their GM content expressed in the same measurement unit as the one established during calibration can be used for a meaningful quality control.

### *7.1 Quality control with CRM available with the adequate GM concentration*

A different CRM from the one used for setting up the calibration curves is used for quality control of the measurement system. This CRM is treated like an unknown sample and analysed using the two calibration curves. The measured value and its standard deviation should be compared to the certified value and its uncertainty as outlined in Section 5.2.2.3 [16].

When using such a matrix CRM for verification, the uncertainty derived from the verification data covers the uncertainty related to the extraction step.

For measurements calibrated in haploid genome ratios the matrix CRM certified for its haploid genome ratio is used for quality control. This CRM is treated as an unknown sample and analysed using the two calibration curves established with the independent calibrant.
Note: CRMs certified for their GM concentration in haploid genome ratios are only available for a few GM events.

### *7.2 Quality control with CRM materials mixed in the laboratory*

Some of the CRMs are not available in the desired GM concentrations and the laboratory may need to produce other GM concentration, e.g. for determining the LOQ and LOD or to have a quality control material close to the GM concentration of interest.

Four situations in which additional GM concentration levels need to be produced by the laboratory can arise:

- the GM and non GM CRMs are only available as pure seed materials (Section 7.2.1);
- the GM and non GM CRMs are only available as pure powder materials (Section 7.2.2);
- the mixed GM CRMs are only available with inadequate GM concentrations (Section 7.2.3);
- the GM and non GM CRMs are only available as extracted DNA solutions (Section 7.2.4).

### *7.2.1 GM and non-GM CRMs available as pure seed materials*

As the material is certified to be pure non-GM and pure GM material, respectively, no homogeneity issues need to be considered for the CRM and a lower sample intake than the minimum sample intake recommended for analysis can be used. Two possible ways are proposed:

#### *7.2.1.1 Mixing at seed level*

Provided the average mass weight of the seeds is similar, the seeds are counted and mixed to achieve the desired composition (1 GM seed with 1999 non-GM seeds for a 0.05 (m/m) %). The whole sample is first ground in a blender. After that, the powder is ground in a mortar to achieve a homogeneous material.
Note: This approach is only feasible for small seeds. At the same time very small seeds may be time consuming to count and the use of balances might be considered. Attention should be paid to the fact that GM seed batches may contain with a certain (low) probability non-GM seeds. Grinding of the samples may lead to different particle sizes or larger particle sizes than required. In this case (or in the case that this cannot be excluded) mixing at DNA level and control of matrix effects by spiking into a non-GMO extract is recommended (Section 7.2.1.2).
Currently only a limited number of CRMs are available as pure seed materials.

#### *7.2.1.2 Mixing at DNA level*

As stipulated in [7] separate portions of non-GM seeds and GM seeds are ground, and the DNA extracted separately from both. The content of the taxon-specific gene for the GM positive (solution *A*) and the GM negative (solution *B*) DNA extract should be measured on the same plate with the same calibration curve. The volume required of solution *B* can be calculated using the following formula (Equation 15):

$$B = \left(\frac{a}{b}\right) \cdot (d - 1)$$

| | |
|---|---|
| $B$ | volume of solution $B$ [µL] (required per µL of solution $A$) |
| $a$ | copy number of the taxon-specific gene in solution $A$ [µL] (GM positive DNA extract) |
| $b$ | copy number of the taxon-specific gene in solution $B$ [µL] (GM negative DNA extract) |
| $d$ | targeted dilution factor (e.g. from 10 % GM to 1 % GM = 10) |

**Example:**

Solution *A* is DNA extracted from a pure GM material (nominal 100 (m/m) % GM) and solution *B* is extracted from a non-GM material (nominal 0 (m/m) % GM). A volume of 5 µL of both solutions is measured by PCR and the relative quantity of the taxon-specific gene determined. As the taxon-specific gene quantity is determined relatively only limited information about the theoretical copy numbers, the average genome size and the zygosity is needed. The theoretical copy number should be estimated to avoid that a sample contains less than 30 copies.

The quantification of the taxon-specific gene gives for example 1000 copies/5 µL (= 200 copies/µL) for solution *A* and 800 copies/5 µL (= 160 copies/µL) for solution *B*. Targeting a dilution factor of 10 (and a nominal concentration of 10 (m/m) % GM), the volume required of solution *B* (in µL) to dilute 1 µL of solution *A* can be calculated using Equation 15:

$B = (200/160) \cdot (10 - 1) = 11.25$ µL, so that 1 µL of solution *A* has to be mixed with 11.25 µL of solution *B*. For practical reasons it is advisable to mix e.g. 100 µL of solution *A* with 1125 µL of solution *B*.

After adding together the two DNA solutions accordingly, the new DNA solution has to be mixed thoroughly.

The mixtures can be used to test the correctness of a measurement result using the diluted extract from a pure GM material and analysing three samples in triplicate, on three different days.

Note: For the estimation of the uncertainty the approach outlined in Section 7.2.2 can be used.

### *7.2.2 GM and non-GM CRMs available as pure powder materials*

As the materials are certified to be pure non-GM and pure GM material, respectively, no homogeneity issues need to be considered for the CRM and a lower sample intake than the minimum sample intake stated on the certificate can be used. Furthermore, the materials have been ground using industrial mills which lead to particles sizes that allow weighing of samples in the mg range without the introduction of too high scatter.

It should be noted that the total DNA content in the pure GM and non-GM material should not be significantly different. This is normally investigated by the CRM producer and reported in the certification report. Additionally, care must be taken that the extraction method employed does not introduce a bias.

In the case that different DNA contents were found in the non-GM and GM powder, the approach as outlined in Section 7.2.1.2 should be used for mixtures at DNA level.

**Example:**

A 1 g sample containing 10 g/kg (or 1 (m/m) %) of a particular GM can be obtained from the following two CRMs:

- GM material certified to contain with 95 % probability > 985 g/kg (> 98.5 (m/m) %) of the GM event;
- non-GM material certified to contain with 95 % probability < 1 g/kg (< 0.1 (m/m) %) of the GM event.

In order to avoid the introduction of a bias due to different water contents of the GM and non-GM material, it is recommended to equilibrate the water content of both powders for 24 h (spread the powder on a dish and expose to the air in the laboratory)[2].

Afterwards the laboratory should use an analytical balance to weigh 0.99 g of non GM powder and 0.01 g of GM powder. The GM mass fraction ($w_{GM}$) is calculated as (Equation 16):

$$w_{GM} = \frac{1000\ (m_{GM}\ \cdot\ p_{GM}\ +\ m_{NGM}\ \cdot\ ip_{NGM})}{m_{GM}\ +\ m_{NGM}}$$

| | |
|---|---|
| $w_{GM}$ | GM mass fraction [g/kg] |
| 1000 | conversion factor from [g/g] to [g/kg] |
| $m_{GM}$ | mass GM powder [g] |
| $p_{GM}$ | purity GM powder [fraction] |
| $m_{NGM}$ | mass non-GM powder [g] |
| $ip_{NGM}$ | impurity non-GM powder [fraction] |

For $m_{GM}$ = 0.01 g, $p_{GM}$ = 1, $m_{NGM}$ = 0.99 g and $ip_{GM}$ = 0 equation 16 results in a GM mass fraction of 10 g/kg. In the next step the laboratory has to estimate the uncertainty associated with the produced 10 g/kg (1 (m/m) %) sample. Four standard uncertainty sources have to be considered:

- uncertainty associated with the weighing of the GM material ($u_{mGM}$),
- uncertainty associated with the purity of the GM material ($u_{pGM}$),
- uncertainty associated with the weighing of the non-GM material ($u_{mNGM}$) and
- uncertainty associated with the impurity of the non-GM material ($u_{ipGM}$).

The uncertainty of the GM and non-GM material purity can be derived from the certificate. The accredited calibration service reported for the analytical balance a relative standard uncertainty of 0.4 %. This uncertainty contributes to the combined standard uncertainty during the weighing of the GM and non-GM material and has to be transformed into an absolute standard uncertainty.

A mass of 0.01 g is weighed for the GM material using a balance with a relative standard uncertainty of 0.4 %. The mass of the GM material ($m_{GM}$) is therefore (0.01000 ± 0.00004) g. The purity of the GM material is certified to be > 985 g/kg (98.5 (m/m) %) with 985 g/kg being the lower limit of the certified 95 % confidence interval. The purity of the GM material ($p_{GM}$) is therefore (1.0000 ± 0.0077), with 0.0077 calculated as (1.000 – 0.985) / 1.96. The division by 1.96 transforms the uncertainty expressed as 95 % confidence interval (corresponding to 1.96 *s*) into a standard uncertainty (corresponding to 1 *s*).

A mass of 0.99 g is weighed for the non-GM material using the same balance with a relative standard uncertainty of 0.4 %. The mass of the non-GM material ($m_{NGM}$) is therefore (0.99000 ± 0.00396) g. The purity of the non-GM material is certified to be < 1 g/kg (0.1 (m/m) %) with 1 g/kg being the upper limit of the certified 95 % confidence interval. The impurity of the non-GM material ($ip_{NGM}$) is therefore (0.0000 ± 0.0005), with 0.0005

[2] In case only intact seed CRMs are available, it is advised to crush the GM and non-GM seeds separately in mortars and to also allow them to equilibrate to the same water content for 24 h.

calculated as 0.001 / 1.96. Again, the division by 1.96 transforms the uncertainty into a standard uncertainty.

The four variables ($m_{GM}$, $p_{GM}$, $m_{NGM}$ and $ip_{NGM}$) are neither all dependent nor independent from each other (equation 16 involves multiplications and divisions and at the same time additions) and combining the standard uncertainties requires the generation of partial differentials [19]. As a practical approach the use of spread sheet software is recommended as outlined in more detail in Annex E2 of [19]. In this approach the GM mass fraction ($w_{GM}$) is calculated by adding to each variable ($m_{GM}$, $p_{GM}$, $m_{NGM}$ and $ip_{NGM}$) its associated standard uncertainty ($u_{mGM}$, $u_{pGM}$, $u_{mNGM}$ and $u_{ipNGM}$), resulting in $m_{GM} + u_{mGM} = 0.01004$ g, $p_{GM} + u_{pGM} = 1.0077$, $m_{NGM} + u_{mNGM} = 0.99396$ g and $ip_{GM} + u_{ipNGM} = 0.0005$.

In the next step equation 16 is resolved four times, each time replacing a different variable by the variable and its associated uncertainty ($m_{GM} + u_{mGM}$, $p_{GM} + u_{pGM}$, $m_{NGM} + u_{mNGM}$, $ip_{GM} + u_{ipNGM}$). From the resulting mass fractions $w_{GM}$ (10 g/kg) is subtracted. The absolute differences are the individual standard uncertainty contributions for each variable:

$u_{(wGM,mGM)} = | w_{GM} + u_{mGM} - w_{GM} | = 0.03960$ g/kg
$u_{(wGM,pGM)} = | w_{GM} + u_{pGM} - w_{GM} | = 0.0765$ g/kg
$u_{(wNGM,mNGM)} = | w_{GM} + u_{mNGM} - w_{GM} | = 0.03944$ g/kg
$u_{(wNGM,ipNGM)} = | w_{GM} + u_{ipGM} - w_{GM} | = 0.5051$ g/kg

The combined uncertainty is calculated by taking the square root of the sum of squares of these values (Equation 17):

$$u = \sqrt{u_{(wGM,mGM)}^2 + u_{(wGM,pGM)}^2 + u_{(wGM,mNGM)}^2 + u_{(wGM,ipNGM)}^2}$$

| | |
|---|---|
| $u$ | combined standard uncertainty |
| $u_{(wGM, mGM)}$ | standard uncertainty in function of $w_{GM}$ and the weighing GM material |
| $u_{(wGM, pGM)}$ | standard uncertainty in function of $w_{GM}$ and the purity GM material |
| $u_{(wGM, mNGM}$ | standard uncertainty in function of $w_{GM}$ and the weighing non-GM material |
| $u_{(wGM, ipGM)}$ | standard uncertainty in function of $w_{GM}$ and the impurity non-GM material |

The combined standard uncertainty is calculated to be 0.51 g/kg. The real GM value of the produced sample and its rounded standard uncertainty [26][3] is therefore estimated to be:

10.0 g/kg ± 0.6 g/kg (1.00 ± 0.06 (m/m) %)

Note: The above calculation concerns the combined standard uncertainty ($u$) as required for instance for trueness control. If an expanded combined uncertainty ($U$) is required, the obtained combined standard uncertainty has to be multiplied with the appropriate coverage factor ($k$) [18].

The DNA should be extracted from the combined materials using a suitable extraction method from the whole 1 g sample, avoiding the need to mix the powder samples homogenously (see co-extraction procedure in Section 7.2.3). Smaller samples can be used to produce a sample, but will be linked to higher uncertainty contributions from the weighing step.

[3] Uncertainties are commonly rounded in such a way that the uncertainty introduced by rounding corresponds to 3-30 % of the uncertainty. In the example given here the value 4.01 is therefore rounded down to 4.

Likewise, attention has to be paid to the DNA extraction step. As further outlined in Section 7.2.3 a co-extraction procedure should be applied in order to avoid the need for a mixing step at powder level, which could easily lead to non-sufficiently homogeneous samples.

### *7.2.3 Mixed GM CRMs available as powder materials with inadequate GM concentrations*

The powders can be used in a so-called co-extraction procedure. As the CRMs are certified GM mixtures homogeneity issues need to be considered and the minimum sample intake has to be respected. In a co-extraction procedure the required amounts of two CRM powders are weighed (using individual minimum weights equal to or above the minimum sample intake), combined and extracted as one DNA extraction sample. There is no need to mix of homogenise the two samples with each other as the whole amount of two samples is used together in the following DNA extraction step. Mixing of the powders (or the use of less than the amount of the two added samples) should not be done as homogeneity cannot be guaranteed.

It should be noted that the total DNA content in the pure GM and non-GM material should not be significantly different. Furthermore, the particle sizes of the non-GM and GM material should not be significantly different as this otherwise introduces a bias on the amount of DNA extracted. These two parameters are normally investigated by the CRM producer and described in the certification report. Additional care must be taken by the laboratory that the extraction method employed does not introduce a bias. This is of utmost importance in cases where the composition of the GM material has been altered by the genetic modification (e.g. starch-modified potatoes).

**Example:**

To get a 1 (m/m) % material while having a 2 (m/m) % and a nominal 0 (m/m) % material, one can co-extract the DNA from a combined sample containing the same amount of both materials. If the minimum sample intake is 100 mg for both materials, the final sample will be 200 mg from which DNA needs to be extracted using a suitable DNA extraction method.
The uncertainty of the GM content of the resulting material is the combined uncertainty of the values stated for the two CRMs used.

Alternatively the approach as outlined in Section 7.2.1.2 can be used for mixtures at DNA level.

### *7.2.4 GM and non-GM CRMs available as extracted pure DNA solutions*

Mixing is required at DNA level to achieve the desired test concentrations.
The example given in Section 7.2.1.2 can be followed.

The estimation of the related uncertainty follows the example given for a mass-based mixture in Section 5.2.2.3.

## *7.3 Additional notes*

(a) For measurements calibrated in mass fractions only one set of CRMs is available. The quality control of measurements expressed in mass fraction is usually limited as the materials used for calibration and quality control are derived from the same batch of CRMs. It is therefore recommended to use the CRM certified for the highest GM concentration for setting up the calibration curve and to use a CRM certified for a lower concentration for quality control or to follow the example given in Section 5.2.2.3. However, the user must be aware that the measurement process cannot be fully controlled in this way.

(b) The use of extracted DNA solutions as CRM for quality control does not cover the DNA extraction step. Checking for matrix effects by spiking into an extract from non-GM material is required. It might be difficult to source a non-GM material which is proven to be completely free of GMOs. However, CRMs certified not to contain a specific GM event could be used to set up a systematic check.

(c) For quality control, also materials analysed and shared by various laboratories could be considered. However, special care has to be taken by the user that the material is sufficiently homogenous and stable.

## *8 Measurement traceability and monitoring of key equipment and methods — ISO/IEC 17025, Sections 5.5.2, 5.6.1 and 5.9.2*

All equipment used within the scope of accreditation and whose accuracy may significantly affect the accuracy or validity of the test result shall be calibrated by a laboratory competent for the task. It is up to individual laboratories to provide formal documented assurance that the requirements for calibration have been met. A competent calibration laboratory may be a laboratory accredited for the specific task by the national accreditation body or by an accreditation body of a country which is a signatory to the European Accreditation Multilateral Agreement. All calibration certificates issued by an accredited laboratory will contain a statement about traceability of the measurement result, including uncertainty and confidence levels.

Non-accredited calibration laboratories may be used whenever such an option is inevitable.

However, the selected laboratory has to prove its competence. Alternatively, and provided that the appropriate technical competence is available within the organisation, it can calibrate its own equipment internally. In such cases, laboratories must develop detailed calibration procedures including estimations on the measurement uncertainties associated with these calibrations.

It should be noted that metrological traceability is also checked by the use of CRMs (Section 7).

For GMO laboratories there are three key pieces of equipment to monitor: balances, thermal cyclers and microlitre pipettes (MPs) [27].

### *8.1 Thermal cycler check*

#### *8.1.1 Maintenance*

In general maintenance should be carried out on an annual basis. The frequency of usage and the cleanness of the incubation chamber (e.g. dust-free handling areas) should be taken into account when defining the required maintenance interval. The maintenance needs to include: cleaning, background testing, general checks and other specific checks depending on the equipment used. Software updates should be considered if recommended by the manufacturer.

The maintenance demands defined by the supplier of the specific instrument have to be met. This includes the messages delivered by the instrument software on maintenance needs. Many laboratories opt for a full instrument maintenance performed by an external company on an annual basis, while specific maintenance procedures are carried out by the laboratory itself with higher frequencies. As long as the demands are met full instrument maintenance and specific maintenance may as well be carried out by the user himself.

After completion of the maintenance it is advisable to analyse an internal quality control sample.

### *8.1.2 Inspection of the performance of the PCR wells*

Inspection of equal amplification and detection across the PCR wells should be carried out at least once per year or after a certain number of PCR runs. The procedure can be the following: a master mix which contains a DNA target is divided up among all wells in order to have approximately 1000 copies of DNA targets per well (a linearised plasmid can for instance be used in conjunction with a dedicated PCR method).

The measured Cq values are imported into an Excel sheet and theoretical copy numbers which should obtained in each well are calculated, taking the number of PCR cycles into account and assuming 100 % PCR efficiency. Mean, standard deviation (*s*) and RSD (RSD [%] = *s* / mean x 100 %) are calculated for each well.

For acceptance, the RSD must be less than or equal to 25 %. The deviation from the mean is determined for each well. It must be less than or equal to 25 %.
If any of the criteria are not fulfilled, a second test is carried out.

Note: Commercial tests can also be used to verify the performance of all PCR wells. They require for instance the proof that the PCR instrument can distinguish between 5000 and 10000 targeted DNA copies with a 99.7% confidence level applying replicate analysis of the two samples. In cases of doubt they could be compared with the outcome of an inspection carried out as outline before.

The described inspection does not verify the correctness of the Cqs measured, but compares the generated Cqs averages. As the variance of the reaction is the critical factor (and not the exact Cqs), this approach is regarded as suitable for the inspection of a qPCR measuring Cqs which are used to express a ratio. The inspection is carried out on an annual basis or depending on the number of PCR runs. However, this inspection cannot replace the controls used on each PCR plate.

### *8.1.3 Background testing and cleaning of the PCR instrument*

Background testing and cleaning of the parts in direct contact with the PCR wells (e.g. thermal block or chamber including the rotor) needs to be carried out in regular intervals taking into account the manufacturer's instructions.

The background test is performed according to manufacturer's instructions (e.g. fluorescence measurement for 2 min at 60 °C of a plate containing 50 µl of ultrapure water per well).

### *8.1.4 Specific checks*

Specific checks need to be carried out and manufacturer's instructions have to be considered. It is up to the laboratory to define if the specific checks are carried out by an external company or by the laboratory itself. They should be carried out in regular intervals taking into account the frequency of use.

The specific check can for instance concern a calibration using a dedicated pre-filled plate, allowing the software to map the position of the wells so that, during instrument operation,

the software can associate increases in fluorescence with specific wells. The halogen lamps should also be checked in accordance with the manufacturer's instructions and depending on the number of PCR runs performed on the device.
A temperature drift monitoring system for thermal cyclers can also be used as periodic validation of the PCR instrument. The accuracy and non-uniformity of the temperature in a PCR cycler are then measured.

## *8.2 Microlitre pipette control*

Each MP needs to be clearly labelled, located and dedicated to a specific use. MPs shall be checked and/or calibrated before use [1, Section 5.5.2].
Inspections are carried out depending on the use and the volume of the MP. They can be based on ISO 8655 [28] and ISO 4787 [29].

MPs should be calibrated at least once a year either by the laboratory or by an external calibration service. For internal checking of volumes of less than 50 µL a five-digit balance is required. A balance connected with software for equipment validation can be used. For example, the check can consist of 10 repetitions at three different levels (e.g. 20, 100 and 200 µL for an MP with an upper volume of 200 µL). The software calculates trueness and repeatability, and indicates the acceptance limits.

It is up to individual laboratories to define the frequency of the MP control.

## *8.3 Internal quality control*

The selection of internal quality control samples, frequency of use and reporting in quality control charts is important. CRMs can be used for internal quality control. It is up to individual laboratories to define the frequency of use of internal quality control samples based on the total number of samples analysed per year. The results obtained from internal quality control samples should be reported on appropriate control charts, using graphical and statistical methods for interpretation. If quality control data are found to be outside of pre-defined criteria, action shall be taken to identify and eliminate the problem.

## *9 Proficiency testing — ISO/IEC 17025, Section 5.9*

The advisory documents *Guidance on the level and frequency of proficiency testing participation* and *ILAC Policy for Participation in Proficiency Testing Activities* [30, 31] give guidance on the level and frequency of proficiency testing (PT) participation. The document does not state a fixed number of tests to be performed within a specified period of time, which still is a requirement from accreditation bodies (EA members) in some countries. Rather, *Guidance on the level and frequency of proficiency testing participation* says that it is up to individual laboratories to define their level and frequency of participation after careful consideration of their other quality management measures. Other quality assurance measures may include (but are not limited to):

- regular use of CRMs,
- comparisons of analysis by independent techniques,
- use of internal quality control measures,
- other inter/intralaboratory comparisons, e.g. analysis of blind samples within the laboratory.

Note: Beside the above, conclusions about the performance of a laboratory could also be drawn from the comparison of data obtained during ILCs organised for method validation or CRM characterisation [30].

Besides the above mentioned quality assurance measures, level and frequency of proficiency testing participation may depend on:

- number of tests undertaken,
- turnover of technical staff,
- experience and knowledge of technical staff,
- source of traceability (use of CRMs or other materials),
- known stability/instability of the analytical procedure.

### *9.1 Level of participation*

The document *Guidance on the level and frequency of proficiency testing participation* acknowledges that it is unlikely to be feasible for a laboratory to participate in a specific PT for every analytical procedure, GM event content and every product. Instead, laboratories should identify groups of products on which the outcome of a PT can serve as a proof of the competence of the laboratory for this specific group of products.

With reference to products to be tested, different products may be included in the same group. Provided that they have a similar behaviour during GM quantification, the number of groups determines the number of product specific PTs a laboratory should participate in.

The minimum frequency of participation for each sub-discipline should be identified by the laboratory.

In all cases, a laboratory must be able to justify the technical arguments for determining the level and frequency of PT participation. The justification should be documented.

## *9.2 Proficiency testing strategy*

According to ISO/IEC 17025:2005 [1, Section 5.9.1], quality control procedures should be planned activities. Once the level and frequency of participation have been established, laboratories should develop a proficiency testing (PT) strategy for which the content and extent of this strategy will depend on the circumstances and scope of the individual laboratory and the availability of PT schemes. The strategy should be a part of the laboratory's overall quality control strategy. The document *Guidance on the level and frequency of proficiency testing participation* recommends that the strategy covers at least one accreditation cycle (period between two full assessments). Furthermore, the laboratory should review the strategy annually and evaluate its appropriateness.

**Example:**

This example concerns a GMO testing laboratory for food, feed and seed, which is accredited for the following testing activities:

- detection and quantification of MON810 maize, MIR162 maize and GTS 40-3-2 soya in feed,
- detection and quantification of MON810 maize, MIR162 maize and GTS 40-3-2 soya in food,
- detection and quantification of MON810 maize, MIR162 maize and GTS 40-3-2 soya in seed.

The laboratory identifies that it uses the same measurement technique (qPCR) for food, feed and seed, but is using a different sample preparation and DNA extraction method for seeds compared to food and feed. Although different GM events may be considered as different parameters, the laboratory can justify and demonstrate equivalence between the qPCR methods for MON810 maize, MIR162 maize and GTS 40-3-2 soya by method verification data. In this case the resulting sub-disciplines would be:

- detection and quantification of GM maize and soya in feed and food
- detection and quantification of GM maize and soya in seeds

## *10 Summary*

The quantification of GMO by qPCR is well established in European GMO testing laboratories even if the measurement principle as such is, compared to more traditional chemical analytical methods, relatively recent. During the last 10 years, a number of GM testing laboratories have obtained an ISO/IEC 17025 accreditation from their national accreditation bodies. However, the ISO/IEC 17025 standard has been interpreted in various ways and a harmonised specific guidance for the accreditation of laboratories performing GMO testing useful for both assessors and testing laboratories was missing.

This document justifies the need for flexible scope accreditation of GMO testing laboratories. Furthermore it covers two major points relevant for both parties. First, it gives a detailed guidance on the interpretation of ISO/IEC 17025 for those undertaking the GMO testing. Guidance is provided for specific ISO/IEC17025 sections that were interpreted differently or needed further explanation.
Secondly, the document outlines on which basis a flexible scope of accreditation can be granted. It explains the different levels where flexibility is possible and provides the additional specific requirements.

This guidance document should allow a better harmonisation of the accreditation process and should facilitate the work of both assessors and GMO testing laboratories.

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**Abstract**

The aim of this guidance document is to facilitate harmonised flexible scope accreditation within Europe, according to ISO/IEC 17025:2005 related to quantitative testing of genetically modified organisms (GMOs) by quantitative real-time polymerase chain reaction (qPCR) for GM events authorised in the EU or which are in the authorisation process.
This document gives guidance to and is intended for laboratories that are considering to acquire a flexible scope of accreditation according to ISO/IEC 17025. At the same time it aims to provide information for assessors involved in the accreditation process of these laboratories.
This guidance document has been written by members of the Task Force (TF) Flexible scope accreditation, which has been initiated by European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (EC JRC-IRMM, Geel, BE). After an extensive commenting phase it has been submitted to the European co-operation for Accreditation (EA) in February 2013 for consideration as an EA guidance document.

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