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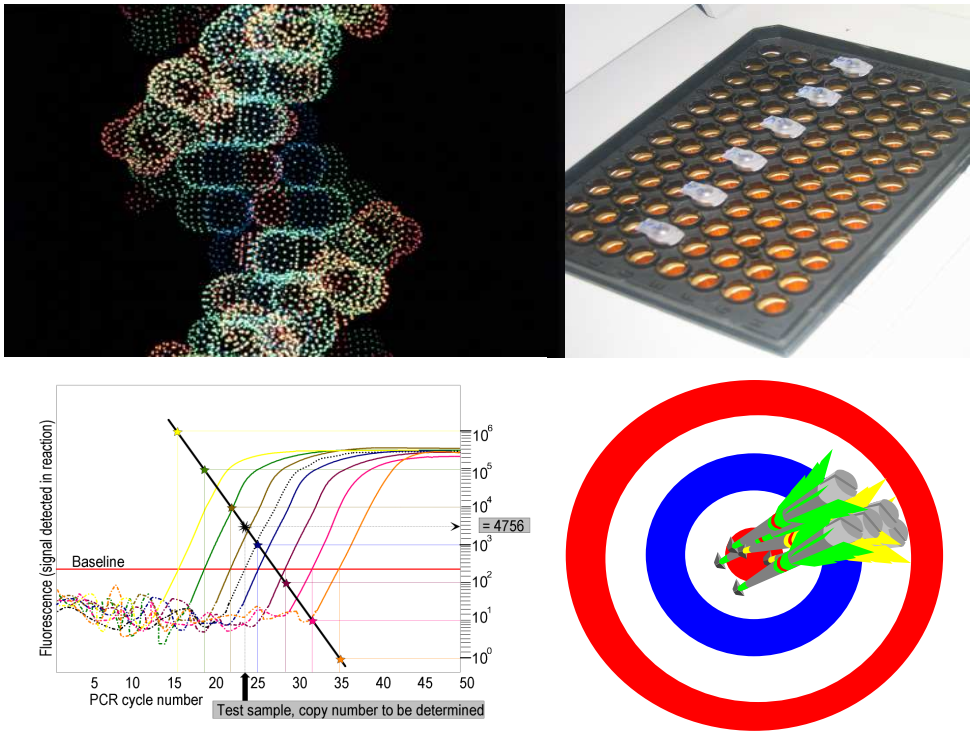

Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing

European Network of GMO Laboratories (ENGL)

2015

Joint Research Centre

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Abstract

The scope of this ENGL document is to provide recommendations on how methods for genetically modified organism (GMO) analysis should be evaluated and validated by the European Union Reference Laboratory for Genetically Modified Food and Feed (EURL GMFF) in the context of Regulation (EC) No 1829/2003 and on the evaluation and validation of such methods used in the context of official controls according to Regulation (EC) No 882/2004.

This document is addressed primarily to applicants submitting detection methods to the EURL GMFF according to Regulation (EC) No 1829/2003 and to National Reference Laboratories (NRLs) willing to develop and propose for validation detection methods, according to Regulation (EC) No 882/2004. More generally, this document is addressed to any institution undertaking method development and optimisation with the final aim of proposing the method(s) for validation to the EURL GMFF.

At present this document refers to polymerase chain reaction (PCR) based methods for the detection, identification and/or quantification of GMOs, in combination with methods for DNA extraction. However, if novel methods (e.g. those using technologies other than PCR) are subsequently developed that fulfil legal requirements, then this document will be amended accordingly.

The document lays down acceptance criteria and performance requirements for DNA extraction and purification methods, PCR methods for the purpose of quantification and PCR methods for the purpose of qualitative detection, being event-specific, construct-specific, element-specific or taxon-specific..

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Contents

1. INTRODUCTION

The scope of this ENGL document is to provide recommendations on how methods for genetically modified organism (GMO) analysis should be evaluated and validated by the European Union Reference Laboratory for Genetically Modified Food and Feed (EURL GMFF) in the context of Regulation (EC) No 1829/2003 [1)] and on the evaluation and validation of such methods used in the context of official controls according to Regulation (EC) No 882/2004 [2)].

This document is addressed primarily to applicants submitting detection methods to the EURL GMFF according to Regulation (EC) No 1829/2003 and to National Reference Laboratories (NRLs) willing to develop and propose for validation detection methods, according to Regulation (EC) No 882/2004. More generally, this document is addressed to any institution undertaking method development and optimisation with the final aim of proposing the method(s) for validation to the EURL GMFF.

The methods fulfilling all the requirements described in the present document are expected to be included in the EU database of Reference Methods for GMO analysis (http://gmo-crl.jrc.ec.europa.eu/gmomethods/).

There is synergy between recommendations made within this document and those of the Codex Alimentarius Commission [3)].

This document takes into account the requirements of the international standards ISO 24276 [4)], ISO 21569 [5)], ISO 21570 [6)] and ISO 21571 [7)].

Method validation is an essential component of the measures that a laboratory accredited according to ISO/IEC 17025 [8)] should implement to allow it to produce reliable analytical data. In some sectors, most notably in the analysis of food and feed, the requirement for methods that have been "fully validated" is prescribed by legislation. 'Full' validation of an analytical method usually comprises an examination of the characteristics of the method in an inter-laboratory method performance study (also known as a collaborative study or collaborative trial). Internationally accepted protocols have been established for the 'full' validation of a method of analysis by a collaborative trial, most notably the IUPAC protocol [9)] and the ISO 5725 [10)]. These protocols/standards require a minimum number of laboratories and test materials to be included in the collaborative trial to validate the analytical method.

The Annex of Regulation (EC) No 1829/2003 states that the EURL GMFF is responsible for the evaluation and validation of methods of analysis in the context of GMO authorisation under Regulation (EC) No 1829/2003. The EURL GMFF is also nominated as European Union Reference Laboratory according to Regulation (EC) No 882/2004. In this context the EURL GMFF is responsible for providing National Reference Laboratories with details of analytical methods, including reference methods. The EURL GMFF is assisted in its activities by the ENGL.

The ENGL recommends that the assessment of methods is undertaken in two distinct phases:

Phase 1 - evaluation of the method performance data in order to decide whether the method is acceptable to undergo full validation (method acceptance criteria).

Phase 2 - confirmation of the fitness for purpose of the method through a full validation study, normally by collaborative trial (method performance requirements)

At present this document refers to polymerase chain reaction (PCR) based methods for the detection, identification and/or quantification of GMOs, in combination with methods for DNA extraction. However, if novel methods (e.g. those using technologies other than PCR) are subsequently developed that fulfil legal requirements, then this document will be amended accordingly.

The document lays down acceptance criteria and performance requirements for DNA extraction and purification methods, PCR methods for the purpose of quantification and PCR methods for the purpose of qualitative detection, being event-specific, construct-specific, element-specific or taxon-specific.

The ENGL and the EURL GMFF endorse the concept of analytical module, as a component of the method; a definition of module is provided below in this document (Annex 1).

Definitions relevant for this document are provided in Annex 1.

2. PHASE 1 - METHOD ACCEPTANCE CRITERIA

This section defines method performance criteria, hereafter "acceptance criteria" to be met before a method is considered ready to enter the validation process (i.e. Phase 2).

The method should be fit for the intended purpose.

In case the method is submitted under the scope of Regulation (EC) No 1829/2003, the applicant should supply the EURL GMFF with:

- evidence that the submitted method fulfils the general principle conditions provided in Annex III of Commission Implementing Regulation (EU) No 503/2013 [11)] and Annex I of Commission Regulation (EC) No 641/2004 [12)]. The PCR module targeting the genetic modification should be event-specific and thus must only be functional with the GMO or GM based product considered and should not be functional if applied to other events already authorised; otherwise the method cannot be applied for unequivocal detection/identification/quantification; and

- evidence that the submitted method meets the acceptance criteria indicated below. Such evidence will include supporting experimental data together with an indication of the reference values and experimental design chosen by the applicant during method testing and optimization; the same evidence should be provided in case of methods intended for validation as reference methods for use in the area of official controls of food and feed, according to Regulation (EC) No 882/2004.

The ENGL recommends that, in case of methods provided by applicants as part of the GMO authorisation process, already validated modules (e.g. DNA extraction, taxon-specific) are used.

In cases of submission of an already validated module(s), the assessment of such module(s) is performed according to parameters described in Annex 2. For the specific case of DNA extraction modules already validated by the EURL GMFF, no further verification is required, provided that the DNA extraction module has been declared fit for the purpose for the same species and on the same matrix.

2.1 Acceptance criteria common to all modules of a method

2.1.1 Applicability

Definition: The description of analytes, sample materials (matrices) and concentrations to which the module can be applied.

Acceptance criteria: The applicability statement should provide information on the scope of the module and include data demonstrating the fitness for purpose of the module with respect to the scope.

For applications submitted under Regulation (EC) No 1829/2003, the whole method should be applicable to samples of the food or feed, to the control samples and to the reference material, which is referred to in Articles 5 (3) (j) and 17 (3) (j) of Regulation (EC) No 1829/2003. The applicant should provide test results of the whole method submitted (i.e. from sample preparation to GMO quantification) on samples submitted in the context of the application (including, where available, reference materials). The EURL GMFF will test the method on the control samples and samples of food and feed and with reference material should this be available during the validation process.

Note: The description should also include warnings to known interferences by other analytes, or inapplicability to certain matrices and situations.

2.1.2 Practicability

Definition: The ease of operations, the feasibility and efficiency of implementation, the associated unitary costs (e.g. cost/sample) of the module.

Acceptance criteria: The module should generally be practicable in line with other applications for a similar purpose. More specifically the module is deemed unacceptable, unless suitable justification is supplied, if:

- it requires a new type of apparatus (not generally available) or expensive equipment; or
- for PCR modules, the temperature-time programme used for the amplification is different between the assay targeting the GMO specific sequence and the assay targeting the taxon-specific sequence; or
- the resources required to perform the analysis (time, workload, reagents, costs) are considerably higher than the resources required to perform other analyses for similar purpose; or
- the total reaction volume exceeds 25 µL.

A DNA extraction module should not involve the use of hazardous chemicals (such as phenol or mercapto-ethanol) if suitable alternative solutions are possible [13].

Other practicability considerations may also deem the method impracticable.

2.2 Acceptance criteria applicable to DNA extraction modules

The aim of a DNA extraction module is to provide DNA of suitable quality and quantity for subsequent analysis. DNA quality depends on the length, structural integrity and physical-chemical purity of the extracted DNA.

As part of the performance assessment of a DNA extraction module, it is recommended to process the given DNA extraction protocol on different days (e.g. 3 days) with an adequate number of test portions (e.g. 6 per day), to obtain suitable data for the evaluation of the module.

The acceptance criteria listed below should be verified on the same working DNA concentration, i.e. the DNA concentration used in subsequent PCR analysis. Dilution will reduce the concentration of the analyte. The structural integrity and purity of the DNA (see below) must therefore be satisfactory at the concentration to be applied in PCR analyses.

The DNA extraction module's criteria should be assessed on a range of representative materials, in line with the intended scope of the module.

In agreement with international guidelines (e.g. ISO 21571 [7], ISO 24276 [4]) the following minimum criteria are used to assess performance of a DNA extraction method.

2.2.1 DNA concentration

Definition: Amount of DNA per volume unit of DNA solution.

Acceptance criterion: The DNA concentration should be appropriate for the subsequent PCR analyses.

Example: if the PCR protocol indicates 40 ng/µL as the DNA concentration of the DNA solution to be added to the master-mix, the average concentration of the DNA extract should be > 40 ng/µL.

Note: The determination and expression of the DNA concentration can be done with reference to mass of DNA (ng/µL) or with reference to the number of copies determined for a representative taxon-specific single copy gene (copies/ µL).

2.2.2 DNA yield

Definition: Total amount of DNA in the extract.

Acceptance criterion: The yield should be at least as much as is required for the subsequent PCR analyses.

The DNA extraction module should provide similar yields for both GM and non-GM material on the same matrix.

2.2.3 DNA structural integrity (size and damage status)

Definition: Breakage of genomic (high molecular weight) DNA into smaller DNA fragments

Acceptance criterion: The minimum size of the majority of DNA fragments should be larger than the size of the amplicon produced by the PCR module used in subsequent analyses. This can be checked by comparison with a reference nucleic acid size marker. The DNA extraction module should not significantly reduce the structural integrity of the DNA or exhibit significant biased selectivity of DNA fragments.

Note: Structural integrity means that the structural characteristics have no negative effect on the detectability of the target DNA sequence. Reduced structural integrity means that the DNA is damaged or has a conformation that reduces the detectability of the target sequence.

Note: Structural integrity is determined for two purposes: 1) in the context of validating a DNA extraction module, to assess whether the module alters the structural integrity and/or has a bias in selectivity; and 2) to verify that a DNA extract has provided DNA fit as template for e.g. quantitative PCR analysis.

2.2.4 Purity of DNA extracts

Definition: The absence of PCR inhibitors in a DNA sample.

Acceptance criterion: the difference (ΔCq) average between the measured Cq value and the extrapolated Cq value of the first diluted sample of the inhibition test should be <0.5 [(measured Cq – extrapolated Cq)] and the slope of the inhibition curve should be in the range of - 3.1 ≤ slope ≤ - 3.6, corresponding to amplification efficiencies of 110% to 90%.

The preferred qPCR module for the inhibition test is a validated taxon-specific reference system (e.g. lectin for soybean DNA).

Note: In case of specific samples from which it may be difficult to extract genomic DNA of high quality (e.g. processed food/feed samples, refined oils, lecithin), a slope of the inhibition curve within -4.1 and 3.1 is acceptable.

2.3 Acceptance criteria applicable to PCR modules

2.3.1 Specificity

Definition: Property of a method to respond exclusively to the characteristic or analyte of interest.

Acceptance criteria: The PCR module should only produce amplification products with the target sequence for which the module was developed. This should be demonstrated by similarity searches against databases (e.g. EMBL, GenBank, Patent, etc.) and with experimental results from testing the module with non-target transgenic events, non-transgenic material and target material. The tests should be conducted with approximately 2500 copies of non-target DNA and with at least 100 copies of target DNA.

A description of the verification of the amplification product should be included in the PCR module. Appropriate techniques are: probe hybridisation, DNA sequence analysis or restriction enzyme digestion or other sequence verification techniques.

Event-specific modules should exclusively detect the targeted GM event.

Element- or construct-specific modules should produce amplification products only with the target sequence.

For taxon-specific modules the absence of allelic variation across a globally representative collection of the taxon should be demonstrated experimentally.

Taxon-specific modules intended for quantitative analysis should target a single DNA copy per allele within the taxon. The absence of copy-number variation across a globally representative collection of the taxon should be demonstrated. The range of variability of Cq values in amplification should not exceed 1 Cq within the taxon.

For multiplex qualitative modules, specificity should be evaluated for each target sequence. The same acceptance criteria as for the corresponding singleplex modules should be applied.

Note: For multiplex qualitative PCR modules, it is recommended to check (e.g. by gel electrophoresis or melting point analysis) if the primers can generate additional amplicons than the expected ones (e.g. a duplex PCR targeting P35S and pat sequences separately, could generate a joint P35S-pat amplicon in addition to the P35S and pat amplicons in case this junction exists in the test material).

The specificity of the detection method should be verified by *in silico* studies against publicly available sequence databases (e.g. EMBL, GenBank, patent, etc.) and experimentally by demonstrating the absence of amplification products when the target sequence specific assay is applied to pure genomic DNA of:

- A representative collection of the closest related taxa;
- The most important food/feed crops.

In case of *in-silico* sequence identities or empirical cross-reactivity of element-/construct-specific modules to other sequences, the fitness for purpose of the method should be demonstrated. This information must be provided in the scope of the method.

Note: When testing the specificity of a PCR module, the potential for obtaining unexpected positive results with reference materials certified has to be considered. Reference materials and other control samples are usually only characterised with respect to presence/absence of a limited set of targets. This means that reference materials and control samples may contain non-declared targets. Non-declared targets can for example be traces of another GMO than the one for which the sample is certified. It is important to be aware of this when applying the reference materials and control samples for example during specificity testing of analytical modules.

Additional acceptance criteria for modules submitted within the scope of Regulation (EC) No 1829/2003:

The module targeting the genetic modification should be event-specific.

The applicant should provide test results on specificity of the event-specific module with at least:

- All the GM events for which reference materials are available at the date of submission of the application for authorisation in the EU
- The event itself and its isogenic line (negative control), with reference to the breeding tree.
- It is recommended to test the specificity also with the events which are in the production pipeline of the applicant and with the events from other GM products, where available.
- Where sample materials are unavailable while insert and event-specific sequence data are available e.g. in a published patent or patent application, the specificity of the event-specific module should be demonstrated by *in silico* analyses.

2.3.2 Dynamic Range

Definition: The range of concentrations over which the module performs in a linear manner with an acceptable level of trueness and precision.

Acceptance criterion: The dynamic range should cover the values corresponding to the expected use. This can be expressed as GMO % or copy number range.

For the combined PCR modules (i.e. GM assay and taxon-specific assay) the dynamic range expressed in relative concentration should comprise at least 0.09% and 4.5%.
The dynamic range of the GM PCR module should be at least between 50 and 2,520 copies.
The dynamic range of the taxon-specific PCR module should be at least between 50 and 56,000 copies, expressed as haploid genome equivalents (HGE). The upper level can be lower in case of species having a large genome size.

Note: The dynamic range is established on the basis of a standard curve tested on a minimum of four concentration levels evenly distributed at least in duplicate.

Dynamic range is not applicable to qualitative methods.

2.3.3 Trueness

Definition: The closeness of agreement between the average value obtained from a large series of test results and an accepted reference value. The measure of trueness is usually expressed in terms of bias.

Acceptance criterion: The trueness should be within ±25% of the accepted reference value over the whole dynamic range of the PCR modules individually (i.e. GM assay and taxon-specific assay) and in combination.

Trueness is not applicable to qualitative methods.

2.3.4 Amplification Efficiency

Definition: The rate of amplification calculated from the slope of the standard curve obtained after a decadic semi-logarithmic plot of Cq values over the DNA copy numbers/quantity.

Acceptance criterion: The average value of the slope of the standard curves should be in the range of - 3.1 ≤ slope ≤ - 3.6, corresponding to amplification efficiencies of 110% to 90%.
The amplification efficiency should be assessed for each module by at least 5 individual runs.
The standard curve should cover the whole dynamic range.

Note: A theoretical efficiency of 100% results in a slope of – 3.32.

Note: The efficiency of the reaction can be calculated by the following equation:

$$Efficiency\,[\%] = (10^{\left(\frac{-1}{slope}\right)} - 1) \times 100$$

Amplification efficiency is not applicable to qualitative methods.

2.3.5 R^2 Coefficient

Definition: R^2 is the coefficient of determination, which is calculated as the square of the correlation coefficient (between the measured Cq value and the logarithm of the copy numbers/DNA quantity of a standard curve obtained by linear regression analysis.

Acceptance criterion: The individual values of R^2 of the standard curves should be ≥0.98.
R^2 should be assessed for each module by at least 5 individual runs. The standard curve should cover the whole dynamic range.

R^2 coefficient is not applicable to qualitative methods.

2.3.6 Precision - Relative Repeatability Standard Deviation (RSDr)

Definition: The relative standard deviation of test results obtained under repeatability conditions. Repeatability conditions are conditions where test results are obtained with the same method, on identical test items, in the same laboratory, by the same operator, using the same equipment within short intervals of time.

Acceptance criterion: The relative repeatability standard deviation should be ≤25% over the whole dynamic range of the PCR modules individually (i.e. GM assay and taxon-specific assay) and in combination.

Note: Estimates of repeatability submitted by the applicant should be obtained on a sufficient number of test results, at least 15, based on ISO 5725-3 [10)].

Note: In case the method is intended to fulfil the requirements of Regulation (EU) No 619/2011 [14)], the applicant should demonstrate a relative repeatability standard deviation ≤25% established on samples containing 0.1% GM related to mass fraction of GM material (for further guidance refer to [15)]).

RSD_r is not applicable to qualitative methods.

2.3.7 Limit of Quantification (LOQ)

Definition: The limit of quantification is the lowest amount or concentration of analyte in a sample that can be reliably quantified with an acceptable level of trueness and precision.

Acceptance criterion: The LOQ should be ≤ the lowest amount or concentration included in the dynamic range (i.e. 0.09% or 50 copies).

The LOQ should be assessed experimentally. Estimates of LOQ should be obtained on a sufficient number of test results, at least 15, by analogy with the requirement set for the assessment of RSDr; this allows estimating the LOQ in conjunction with the assessment of RSDr.

LOQ is not applicable to qualitative methods.

2.3.8 Limit of Detection (LOD)

Definition: The limit of detection is the lowest amount or concentration of analyte in a sample, which can be reliably detected, but not necessarily quantified.

Acceptance criterion:
For combined modules the LOD should be < 0.045% with a level of confidence of 95%, ensuring ≤ 5% false negative results.

For individual modules the LOD should be < 25 copies with a level of confidence of 95%, ensuring ≤ 5% false negative results.

Note: In order to reach the required level of confidence, a suitable number of replicates should be tested. As an example, the number of replicates tested per amount or concentration may be 60, with the LOD set at the lowest concentration yielding at least 59 positive results (Cochran 1977 [16)], Zar 1999 [17)]).
The amounts tested may include approximately 20, 10, 5 and 1 copy. As the probability distribution suggests that, in case of 60 replicated tests, 1 copy should give approximately 36% negative results (CI95%: 24-49), this should be taken into account to verify that the copy numbers of the dilution series are approximately correct [18)].
The use of a different approach should be supported by sound statistical evidence ensuring that the level of confidence required is reached.

Additional acceptance criteria for multiplex qualitative PCR modules:

For multiplex qualitative PCR modules an asymmetric LOD (LODasym) should be determined.

The LODasym is determined by testing the analyte target at low amount or concentration (corresponding or close to the absolute LOD) in the presence of increasing amounts or concentrations of the other target(s) in the multiplex assay.
The LODasym is expressed as the minimum ratio between the copy number of the tested analyte target and the copy number level of the other target(s) for which the analyte target can still be detected with a level of confidence of 95%, ensuring ≤ 5% false negative results.

Note: as an example, in a duplex PCR module, if 20 copies of the target sequence are detected with a level of confidence of 95%, in presence of 20,000 copies of the other target, the LODasym is then a ratio below 1:1000.
In case of multiplex PCR modules, the LODasym can be determined by testing e.g. 20 copies of each target sequence in presence of a background of all other targets summed at the level of 20,000 copies.
As described for the LOD the required level of confidence can be achieved with a suitable number of replicates that should be tested. As an example, the number of replicates tested per amount or concentration may be 60, with the LODasym set at the lowest concentration of the target in presence of the other target at the higher concentration yielding at least 59 positive results (Cochran 1977 [13)], Zar 1999 [14)]).

2.3.9 Robustness

Definition: The robustness of a method is a measure of its capacity to remain unaffected by small, but deliberate deviations from the experimental conditions described in the procedure.

Acceptance criterion: the method should provide the expected results when small deviations are introduced from the experimental conditions described in the procedure.

Note: for a PCR module, the following factors should be tested:

- thermal cycler (brands and models)
- master mix (final concentration)
- reaction volume
- primers concentration
- probe concentration
- annealing temperature

For quantitative modules, based on the acceptance criterion of ≤25% for the relative repeatability standard deviation (RSD_r) and trueness, the RSD_r and trueness calculated for a combination of changes should not exceed 30%.

For qualitative modules all replicates should give positive results.

For quantitative methods/modules the target amount/concentration to be tested should be at the LOQ.

For qualitative modules the target amount/concentration to be tested should be 3 times the LOD.

Examples for robustness tests are provided in Annex 3.

3. PHASE 2 - METHOD PERFORMANCE REQUIREMENTS

The purpose of a collaborative trial is to verify the transferability and performance of a method among laboratories, according to the principles of either the IUPAC harmonised Protocol [14)] or ISO 5725 [14)]. This section sets and describes the criteria according to which the results of collaborative trials are evaluated.

3.1 Precision - Relative Reproducibility Standard Deviation (RSD_R)

Definition: The relative standard deviation of test results obtained under reproducibility conditions. Reproducibility conditions are conditions where test results are obtained with the same method, on identical test items, in different laboratories, with different operators, using different equipment. Reproducibility standard deviation describes the inter-laboratory variation.

Acceptance criterion: The relative reproducibility standard deviation RSD_R should be <35% over the whole dynamic range. However, at relative concentrations <0.2% or at an amount <100 copies RSD_R values <50% are deemed acceptable.

Note: RSD_R is usually calculated only for quantitative methods. However, various authors proposed protocols allowing the determination of precision of qualitative methods by interlaboratory studies [16)] [17)] [18)]. These protocols make use of the notion of Probability of Detection (POD).

3.2 Trueness

Definition: The closeness of agreement between the average value obtained from a large series of test results and an accepted reference value. The measure of trueness is usually expressed in terms of bias.

Acceptance criterion: The trueness should be within ±25% of the accepted reference value over the whole dynamic range.

Trueness is not applicable to qualitative methods.

3.3 False positive rate (type I error rate)

Definition: The probability *α* of making a type I error (scoring a false positive).

Note: A false positive occurs if the test result is classified positive (GM target or a specific GMO is detected) when the actual condition is negative (GM target or the specific GMO is absent).

Acceptance criterion: 5% ≥ α.

α = 100 x number of misclassified known negative samples/total number of known negative samples

Note: False positives may be caused by errors in a participating laboratory or are sometimes observed when reference materials are used as negative controls, because the reference material is not certified for presence/absence of the target sequence in question.
In such cases the positive test result is not necessarily a type I error and the coordinator of the validation study should investigate and discuss the reasons for the occurrence of the unexpected positive results.

3.4 False negative rate (type II error rate)

Definition: The probability β of making a type II error (scoring a false negative).

Note: A false negative occurs if the test result is negative (GM target or a specific GMO is not detected) when the actual condition is positive (the GM target or the specific GMO is present at a concentration ≥ LOD).

Acceptance criterion: 5% ≥ β at a GM target concentration ≥LOD.

β = 100 x number of misclassified known positive samples/total number of known positive samples

3.5 Probability of Detection (POD)

Definition: The probability of a positive analytical outcome for a qualitative method for a given matrix at a given concentration. It is estimated by the expected proportion of positive results for the given matrix at the given analyte concentration.

The assessment is an optional approach and can be done in a collaborative study for a qualitative method (Wilrich 2010 [19)], Wehling 2011 [20)], Macarthur 2012 [21)], Uhlig 2015 [22)]). To obtain data for determination of the POD, different laboratories measure replicate DNA samples at increasing GM target concentrations in the range close to the absolute LOD. The POD can be assessed in addition to, but not replacing, false positive and false negative rate and provides additional information on the performance of the qualitative module.

With an appropriate design of the collaborative study, POD response curves can be calculated to provide an overview of the performance of the PCR module. This response curve should be compared to the “ideal curve” calculated on basis on the underlying probability (Poisson) distribution of the GM target across the concentration series. The POD can be also calculated for all laboratories or separately for each laboratory to identify outliers.

The POD can be used to calculate the average GM target concentration at POD = 0.95 (95% probability of detection).

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ANNEX 1: DEFINITIONS RELATING TO TARGETS AND APPLICATION OF PCR MODULES

The GMO method, as understood by the ENGL (see e.g. the ENGL guidance document on measurement uncertainty for GMO testing laboratories, Trapmann *et al*., 2007), is the full procedure, from sample preparation to data interpretation and reporting, used to determine the presence, identification and quantification (when necessary) of GMOs in food, feed or seed samples and providing a measurement result.

The method is defined as qualitative or quantitative, according to the purpose of its use within the analytical procedure:

Qualitative method = method of analysis whose response is either the presence or absence of the target DNA sequence(s) in a sample.

Quantitative method = method of analysis whose response is the quantity of the target DNA sequence(s) in a sample.

Singleplex PCR method = method of analysis detecting a single target DNA sequence in one reaction.

Multiplex PCR method = method of analysis simultaneously detecting two or more DNA sequences in one reaction.

The method may consist of several analytical steps (sample preparation, DNA extraction/purification, qPCR and data evaluation) each of which is represented by one or more modules. The same module can be used in different methods.

This document covers the modules for DNA extraction/purification and DNA analysis by e.g. qPCR.

This document covers also multiplex PCR modules (targeting two or more independent DNA sequences in the same tube) intended for qualitative analyses.

The PCR modules applied for GMO detection, identification and quantification are divided into the following categories with respect to the desired target specificity:

Taxon-specific module = a module that detects a sequence known to be specific for the target taxon.
Note: the target sequence should be consistently present in the target taxon and absent in other taxa. In case the module is intended for quantitative purposes, presence of the target sequence as a single copy in the genome of the taxon is preferred.

Element-specific module = a module that targets a single discrete DNA sequence.
Note: The genetic element can for example be a promoter, a terminator, an intron or the coding part of a gene. Elements are often derived from naturally occurring viruses, bacteria, plants, etc. A positive test result with an element-specific module is sometimes but not always a confirmation of the presence of GMO-derived DNA in the sample.

Construct-specific module = a module that targets an inserted DNA sequence composed of at least two elements that do not naturally co-exist in this conformation, and where the 5' and 3' end of the sequence are derived from two separate elements.

Event-specific module = a module that targets a sequence unique to a single genetic modification event.
Note: The event-specific sequence is the signature of a particular GMO, and is created *de novo* when the construct is integrated into the recipient genome. Event-specific sequences are usually but not always the integration-border regions i.e., the fusion sequence composed of the terminal base pairs of the inserted DNA and the adjacent base pairs of the recipient host genome at the insertion locus. A construct-specific

target cannot be event-specific because the same construct can be used repeatedly to develop new genetic modification events.

The applications of these modules include:

Screening = a test with the purpose to rapidly and reliably sort samples into groups that will facilitate and potentially reduce the required subsequent analytical work and results interpretation.

Screening can use modules with all types of target specificity (as defined above) which can be qualitative or quantitative.

Identification = a test with the purpose to verify if a specific GMO (event) is present or not detectable in a sample.
Note: Identification can be performed simultaneously with quantification, but identification is always a qualitative characteristic.

Quantification = a test with the purpose to determine the quantity of target sequence(s).
Note: The quantity is determined with reference to a specific unit, for example the number of target sequence copies or the mass of GM material. Reporting of a relative GMO concentration is always based on the relative relationship between two measured quantities, e.g. the quantity of a taxon-specific target and the quantity of a GMO specific target [23)].

ANNEX 2 – METHOD VERIFICATION CRITERIA

This annex sets and describes the criteria to be met when an already fully validated method is considered ready to enter the EURL GMFF validation process. These criteria, hereafter "*Method Verification Criteria"*, are used to evaluate if the performance of a method is sufficiently satisfactory in order to proceed with the EURL GMFF method verification process.

The method provider should give:

- evidence that the submitted method meets the acceptance criteria indicated below. Such evidence will include the supporting experimental data and all the necessary information, together with indication of the reference values and experimental design chosen by the method provider during method testing and optimisation;

for methods submitted in accordance to Regulation (EC) No 1829/2003:

- evidence that the submitted method fulfils the general principle conditions provided in Annex III of Commission Implementing Regulation (EU) No 503/2013 and Annex I of Commission Regulation (EC) No 641/2004: the method should detect and quantify the specific GM event in a product; it should be event-specific and applicable to the notified (or otherwise relevant as determined by the EU RL GMFF) material as well as to the samples of the food and feed, and their control samples;

- reference to previous application(s) including details and relevant information from the detection method to be referred to.

In this section the characteristics and their definition used for method evaluation refer to those described and defined within Phase 1.

For the assessment of the following characteristics the method provider should refer to existing data demonstrating the positive evaluation of the detection method:

Applicability
Practicability
Specificity
Limit of Detection (LOD)
Robustness

For methods submitted in accordance to Regulation (EC) No 1829/2003 the following characteristics should be evaluated by the method provider against the samples of the food and feed, and control samples related to the new application. The evaluation should be performed as described in sections dealing with Phase 1:

Dynamic Range
Trueness
Amplification Efficiency and R^2 Coefficient
Repeatability Standard Deviation (RSD_r)
Limit of Quantification (LOQ)

For any other method, the above characteristics should be evaluated by the method provider on samples suitable for the intended scope of the method.

ANNEX 3 – PROCEDURE FOR ESTIMATING THE ROBUSTNESS OF A PCR MODULE

To obtain experimental data on the robustness of a PCR module, tests should be conducted using different brands/models of thermal cyclers, different concentrations and volumes of master mixes, different annealing temperatures and different primer and probe concentrations. The following procedure describes an example for robustness tests of qualitative PCR methods [1)].

Procedure:

A multifactorial experiment design is recommended [2)]. Relevant factors and appropriate PCR assay deviations should be tested at least at two different conditions (Table 1). Some possible combinations of these conditions are shown in Table 2. Each combination should be tested at least in 3 PCR replicates (Table 3). Two PCR runs are required (run 1 at +1°C and run 2 at -1°C) on each brand/model of thermal cycler.

The target sequence copy number used in the tests should be at the LOQ of the PCR module. If the LOQ is not known, a simplified estimation can be done by multiplying the LOD by three. The target DNA should be diluted in 5 ng/µL non-target DNA.

Table 1. Example of conditions tested

Factor	Condition 0	Condition 1
Thermal cycler (brand/model)	A	B
Master mix concentration	unchanged	- 10%
Primer concentration	unchanged	- 30%
Probe concentration	unchanged	- 30%
Master mix volume (total of 25 µL)	19 µL master mix + 5 µL sample DNA	21 µL master mix + 5 µL sample DNA
Annealing temperature	+1°C	-1°C

Table 2. Multifactorial design of the conditions outlined in table 1

Factor	Combination							
	1	2	3	4	5	6	7	8
Thermal cycler	0	0	0	0	1	1	1	1
Master mix	0	0	1	1	0	0	1	1
Primer concentration	0	1	0	1	0	1	0	1
Probe concentration	0	1	1	0	1	0	0	1
Master mix volume	0	0	1	1	1	1	0	0
Annealing temperature	0	1	0	1	1	0	1	0

The experiment design allows that any pair of factors (e.g. master mix and primer concentration) is represented at least twice in all four possible factor combinations (0/0, 0/1, 1/0, and 1/1). Thus, the design will also detect possible factorial interactions.

Table 3. PCR setup scheme

Factor	Combination							
	1	2	3	4	5	6	7	8
Cycler	A	A	A	A	B	B	B	B
Master mix	unchanged	unchanged	- 10%	- 10%	unchanged	unchanged	- 10%	- 10%
Primer conc.	unchanged	-30%	unchanged	-30%	unchanged	-30%	unchanged	-30%
Probe conc.	unchanged	-30%	-30%	unchanged	-30%	unchanged	unchanged	-30%
MM vol. / Anneal. temp.	19 µL	19 µL	21 µL	21 µL	21 µL	21 µL	19 µL	19 µL
+1°C (PCR run 1)	Replicate 1		Replicate 1			Replicate 1		Replicate 1
	Replicate 2		Replicate 2			Replicate 2		Replicate 2
	Replicate 3		Replicate 3			Replicate 3		Replicate 3
-1°C (PCR run 2)		Replicate 1		Replicate 1	Replicate 1		Replicate 1	
		Replicate 2		Replicate 2	Replicate 2		Replicate 2	
		Replicate 3		Replicate 3	Replicate 3		Replicate 3	

Note: If negative PCR results are observed for any combination(s) they should be repeated once. If the negative results are confirmed in the second test, the outcome indicates insufficient robustness of the PCR method.

References for Annex 3

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