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Guidelines on the Validation of Modifications of GMO Reference Methods

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Abstract

These guidelines explain how laboratories should assess and validate modifications to EU reference PCR methods used for detecting and quantifying genetically modified organisms (GMOs), so that methods remain functional and compliant to Annex III of Regulation (EU) No 503/20132 and to the criteria listed in the ENGL guidance document 'Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing'. They were prepared by the ENGL Working Group on Update of Methods and revised by the EURL GMFF to support practical implementation in laboratories.

The document explains that validated GMO detection methods may need reassessment because of evolving Minimum Performance Requirements (MPR), technological developments and the unavailability of original reagents. It provides recommendations on which performance parameters should be reassessed for common modifications. For each case, the document identifies how method performance parameters should be re-assessed when modifications are implemented.

Foreword

The ENGL agreed on the need to establish a dedicated working group with the task to develop recommendations and actions to be undertaken when a (validated) method is modified and updated, compared to the original method development.

The original mandate had foreseen the establishment of procedures for updating methods (e.g. by applicants at the time of renewal of an authorisation), with the involvement of the EURL GMFF and a monitoring role for the working group. Before the publication of the guidelines, in March 2025 the ENGL Steering Committee acknowledged that most methods were already updated (e.g. by method developers in the frame of stack applications) and aligned with the current MPR. Hence, there was no pertinent need for developing a new formalised evaluation procedure for methods that have small technical issues or for renewal applications. Nevertheless, the guidance for laboratories when experiencing an issue with a validated method was deemed useful to publish, in a simplified version.

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Authors

The Working Group on Update of Methods (WG-UpMeth) of the European Network of GMO Laboratories (ENGL) drafted the present Guidelines. The working group was chaired by Roberta Onori (Istituto Superiore di Sanità ISS, IT). The guidelines were thereafter revised by the EURL GMFF, with the coordination of Marco Mazzara (European Commission, Joint Research Centre, IT), to focus on their practical implementation in the laboratories.

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1 Introduction

The placing on the EU market of food and feed consisting of, containing or produced from genetically modified organisms, also referred to as genetically modified food and feed, is subject to authorisation according to Regulation (EC) No 1829/2003[1)]. The authorisation granted in accordance with the procedure referred to in this Regulation lasts for ten years and is renewable, in accordance with Art. 11 and 23. The requirements for authorisation include the provision of a detection method. The European Union Reference Laboratory for Genetically Modified Food and Feed (EURL GMFF) performs the scientific assessment and validation of detection methods for GM Food and Feed as part of this EU authorisation procedure. The method, once validated by the EURL GMFF, must remain functional for the entire duration of the authorisation period.

Regulation (EU) No 503/2013[2)] establishes that the method shall fulfil the specific requirements stated in Annex III, referring to the operational procedures of the validation process that are made available by the EURL GMFF. The detection method must fulfil the requirements and meet the acceptance criteria listed in the ENGL guidance document entitled 'Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing'[3-4)] (hereinafter referred to as 'MPR'). This document is in line with the ISO[5)] and Codex Alimentarius[6)] requirements for nucleic acid-based methods in the domain of GMOs and constitutes the reference text for the assessment of PCR measurement methods.

Therefore, it is useful to provide guidelines for control laboratories and methods developers to verify if a method is still functional and/or compliant with the MPR[3)], e.g. during the validity of the authorisation.

Over the years, different versions of the MPR have been published (2004, 2008, 2015[3)], 2023[4)]). Some of the requirements in the latest MPR[3)] have been further elaborated or modified compared to former versions. Furthermore, technological advances in real-time PCR platforms and unavailability of reagents could have an impact on the applicability and practicability of originally validated methods. Therefore, performance parameters of a method validated several years ago may need to be re-assessed by the EURL GMFF or by the laboratory using the methods.

It is important to ensure that the validated methods (including DNA extraction methods) continue to perform acceptably according to the current scientific understanding and requirements.

2 Definitions

In this document, the criteria and definitions used for method evaluation refer to those described and defined in the latest version of the MPR[3], in 'Verification of analytical methods for GMO testing when implementing inter-laboratory validated methods'[7] and in ISO 16577:2016 'Molecular biomarker analysis - Terms and definitions'[8].

The term 'single-laboratory' validation, meaning 'in-house' validation, is used throughout this document for consistency with Regulation (EU) No 503/2013, Regulation (EC) No 1981/2006[9] as amended by Regulation (EU) No 120/2014[10] and Recommendation (EC) 787/2004[11].

The ENGL and the EURL GMFF endorse the concept of 'analytical module', as a component of the method; the definition of module in this document is the one provided in Annex 1 of the MPR[3].

The following definitions refer to this document:

PCR measurement method [a]

Description of a logical organisation of operations based on the combination of an enzymatic *in vitro* amplification of a specific DNA fragment with the quantification of the amplified PCR product. Thereby the measurement model [b] and the experimental conditions, which ensure the performance of the method in compliance with the Minimum Performance Requirements defined by the ENGL, are specified.

PCR measurement procedure

Detailed description of the experimental conditions that can be changed without affecting the Minimum Performance Requirements defined by the ENGL.

Practicability [3]

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

Validation of method [12]

Provision of objective evidence that a given item fulfils the specified requirements that are adequate for an intended use (ISO 17025 section 3.9).

Verification of method [12]

Provision of objective evidence that a given item fulfils specified requirements (ISO 17025 section 3.8).

[a] The term "PCR method" is used in this document as short term for PCR measurement method.

[b] Measurement model: mathematical relation among all quantities known to be involved in a measurement (16577:2016 Ed. 1 Molecular biomarker analysis - Terms and definitions).

Non-functional method

A validated method is considered as non-functional when its ability to identify and quantify the analyte of interest, i.e. the GM-target, in an event-specific manner is no longer achieved or when one or more required reagents are no longer available on the market and cannot be substituted with equivalent products.

3 General considerations on the evaluation of method modifications

The document on 'Verification of analytical methods for GMO testing when implementing interlaboratory validated methods' mentions that "*as a matter of principle, a method should be implemented as validated in interlaboratory trial, without introducing modifications. If single elements (e.g. the brand or the composition of ready-to-use reaction mix or Taq polymerase, the PCR reaction volume, the primers and probe concentrations, and/or PCR cycling parameters) are modified, additional performance parameters should be experimentally assessed (e.g. specificity and robustness)*".

ISO/IEC 17025:2017[12)] details that:

- "*When changes are made to a validated method, the influence of such changes shall be determined and where they are found to affect the original validation, a new method validation shall be performed" (7.2.2.2);*

- *"The performance characteristics of validated methods, as assessed for the intended use, shall be relevant for the customers' needs and consistent with specified requirements" (7.2.2.3).*

According to the definition of PCR measurement method, the methods remain fit for purpose if the measurement model and the experimental conditions keep the performance of the method within the MPR[3)] defined by the ENGL. Modifications of the PCR measurement procedure can be done if verified by the laboratory and the performance of the method is still compliant with MPR[3)].

Consequently, National Reference Laboratories (NRLs) and GMO control laboratories have to verify that the methods as applied in their laboratory environment (equipment, reagents, staff) perform according to the criteria laid down in the latest MPR[3)].

For practicability reasons, modifications to validated methods can be introduced. However, modified methods shall always meet the established minimum performance requirements in force.

A survey among ENGL laboratories[13)], launched in July 2015 by the EURL GMFF, confirmed that almost all official control laboratories (98%) are using EU reference methods (http://gmo-crl.jrc.ec.europa.eu/gmomethods/) for performing quantitative analysis of GMOs. However, the survey also confirmed that almost half of the laboratories have modified the procedures for quantitative GMO analysis. In more than 30% of these methods, the PCR master mix is modified. The reaction volume is the second most represented alteration made to quantitative PCR assays. In some cases, master mix, reaction volume and amplification reaction conditions have been harmonised for all the methods used in one laboratory. Since laboratories using modified methods in general achieve good performance scores in proficiency tests organised by the EURL GMFF, these modifications do not seem to affect the analytical accuracy or sensitivity of the original procedures.

This document provides information on performance parameters to be assessed when a modification of a validated method is introduced. The modification may be introduced by a laboratory for practicability reasons or, upon request of the EURL GMFF, by a method developer in order to maintain the method's functionality and MPR[3)] compliance, thus prolonging the validity of the authorisation, e.g. at the renewal stage or at any time during the authorisation period.

4 Actions recommended

Table 1 summarises possible modifications of validated methods and recommended actions in line with the ENGL method performance requirements. These are discussed in detail in the following paragraphs.

The ENGL documents 'Verification of analytical methods for GMO testing when implementing interlaboratory validated methods' and the MPR[3)] further specify the practical experimental settings for the single laboratory reassessment of the performance requirements.

4.1 Specificity

For each newly developed method (event-specific and taxon-specific (reference) module) the specificity of the method has to be assessed by the method developer, providing *in silico* and experimental data demonstrating that the expected specificity is obtained. In case of methods developed for authorisation purposes according to Regulation (EC) No 1829/2003, these data are evaluated by the EURL GMFF in a stepwise procedure, including bioinformatics analysis on the specificity of the amplification modules.

If the specificity of a validated method is compromised due to changes in analytical conditions, such as the unavailability of a critical component, and suitable alternatives are not available; or if the specificity is compromised due to the emergence of a new analyte that cross-reacts with the method, a new method should be developed and validated (in case of methods developed for authorisation purposes according to Regulation (EC) No 1829/2003, a new method is developed by the applicant and validated by the EURL GMFF). This is because the specificity of a PCR method is provided by the unique oligonucleotide sequence of the primers that cannot be modified without *de facto* producing a new method.

4.2 Robustness

Robustness is assessed against the version of the MPR[3)] applicable at the time of development of the method. In case of modifications of some parameters of the method (see Table 1), the method developer or the user of the method should assess that the robustness, tested at the time of method development, is still fulfilling the requirements laid down in the latest MPR[3)].

4.3 Calibration curve validated with ΔCq approach

Some methods that used the ΔCq approach have been validated in the past[14), 15), 16)]. This approach entails that a single calibration curve for the GM module is run with decreasing concentrations of the GM-target (for example 5.0%, 1.0%, 0.5%, 0.1%) in a fixed amount of total species DNA. The ΔCq calibration curve results from the subtraction of the Cq value of the reference module from the Cq value of the GM module for each respective calibration standard. The use of the ΔCq method is only valid if the amplification efficiencies of the target taxon-specific module and the event-specific module are very similar (ISO 21570:2005[17)]; ENGL guidance document on flexible scope accreditation[18)]). A major limitation of this methodology is that it lacks a calibration curve for the reference gene and for this reason the dynamic range of the taxon-specific PCR module is not compliant with the current MPR[3)] acceptance criteria (dynamic range at least between 50 and

56,000 copies expressed as haploid genome equivalents). Therefore, ΔCq methods validated as such in previous years should be adapted as they do not comply with the latest MPR[3].

For PCR methods that were originally validated with the ΔCq approach, it is recommended to perform a single-laboratory validation according to the current MPR[3], applying the two standard curves approach. The performance parameters to be assessed are listed in Table 1.

4.4 Different annealing temperature between the GM and taxon-specific reference modules

When the GM- and taxon-specific reference modules have different annealing temperatures, the quantification requires the two PCR modules (GM and taxon-specific module) to be run on separate plates, with an increase in analytical costs. For practicability reasons, it would be beneficial to apply the same amplification profile for all qPCR methods. Lowering the annealing temperature of a PCR method may affect the specificity of the method. If the annealing temperature of the module is increased, the new conditions are more stringent for primer annealing, therefore, it is not necessary to test the specificity again.

When the annealing temperature of a method is decreased, it is recommended to verify the specificity of the method in the laboratory. The performance parameters to be assessed are listed in Table 1.

4.5 Reaction volume exceeding 25 µL

The current MPR[3] requires methods to be run in a maximum reaction volume of 25 µL in order to minimise the reaction costs and to make sure that methods are compatible with the PCR platforms in use.

If a laboratory wants to run a method in a smaller reaction volume than the volume with which the method was previously validated, it is recommended to perform a single laboratory validation. Performance parameters to be assessed are listed in Table 1.

4.6 Issues with reagents

In some cases, reagents used during validation of a DNA extraction or PCR method may be taken off the market or are replaced by other reagents. Changing to other components of a DNA extraction method or other PCR reagents may have effects on the performance of the method.

Some PCR reagents like the master mix, the reaction buffer, the enzyme, surfactants and others, may be phased out due to commercial strategies of the producing companies. Primer and probe sequences cannot be altered as they are part of the core components of the method, however,

fluorophore/quencher chemistry may need to be changed to enable more sensitive detection or to allow multiplexing [c].

Other issues may derive from changes in the pH of the buffer or batch-to-batch variability. Complex protocols involving the combination of many separate reagents can result in error-prone laboratory operability and reduced practicability.

Since it is not possible to exclude that modifications in the master mix composition have an influence on the specificity of the method, re-assessment of the specificity of the two PCR modules is recommended. For taxon-specific modules, it is recommended to re-assess the specificity on the main GM crops (see MPR[3)] for details on how to assess specificity).

Both for DNA extraction and PCR modules in which a critical reagent is replaced by another one, it is recommended to perform a single-laboratory validation. Performance parameters to be assessed are listed in Table 1.

4.7 Taxon-specific reference module

Ideally, a reference gene should occur as a single copy per haploid genome[19)]. Multiple copies of a reference gene may indeed cause a bias in quantification when using copy numbers as the unit of measurement (e.g. using dPCR) and converting the results into a GM mass fraction[20-21)].

Note that this bias will not occur when using a CRM certified in GM mass fraction as calibrant and using the mass fractions as unit of measurement.

Some currently used taxon-specific modules may target multiple gene copies. As an example, the acyl carrier protein (*acp*1) used for cotton can potentially target two regions on chromosome 15, and the *cruA* gene used for oilseed rape is present on both the A and C genome, while the GM event is present on only one genome.

In addition, for practicability reasons, the use of the same taxon-specific modules for all event-specific methods developed for a species may increase the efficiency of GMO analysis in the laboratory. Considering the modularity of GMO methods, changing to a different taxon-specific module that was previously validated for the species does not require additional experimental validation.

It is recommended to replace the taxon-specific modules targeting a *multiple-copy gene* with a 'single copy' taxon-specific module that is already validated. If a validated 'single copy' taxon-specific module is not available, a single laboratory validation of the *combined* GM/reference modules should be performed. The performance parameters to be assessed are listed in Table 1.

[c] Please consult the ENGL report "Guidance document on multiplex real-time PCR methods" for more information on the choice of reporters and quenchers" (https://gmo-crl.jrc.ec.europa.eu/ENGLabs#inline-nav-engl-reports)

4.8 Dynamic range

The current MPR[3)] requires that the dynamic range for the combined PCR modules (i.e. GM module and taxon-specific module), expressed either as GM copy number ratio or GM mass fraction, ranges at least from 0.09% to 4.5%. Some methods validated in the past have not been tested over the full range. Moreover, in specific cases it may be useful to extend the dynamic range of a method at the low or high end or shift it to a different range.

When the dynamic range of a method is modified compared to the validated range, it is recommended to perform a single laboratory validation over the new dynamic range. The performance parameters to be assessed are listed in Table 1.

Table 1. Actions and performance parameters to be assessed when modifying a method

Reason for modification	**Actions**	**Performance parameters to be assessed**
Method validated with ΔCq approach	Use of two calibration curves, i.e. for taxon- and GM-module	Dynamic range, trueness, amplification efficiency, R^2 coefficient, RSD_r, LOQ (Annex II MPR[3)])
Different annealing temperatures for the GM and taxon-specific modules	Harmonisation of annealing temperatures between the two modules of the event-specific detection method	Dynamic range, trueness, amplification efficiency, R^2 coefficient, RSD_r, LOQ (Annex II MPR[3)]). Specificity in case of decreased annealing temperatures
Reaction volume > 25 µL	Change to volume ≤ 25 µL	Dynamic range, trueness, amplification efficiency and R^2 coefficient, RSD_r, LOQ (Annex II MPR[3)])
Phased-out reagents as components of the reaction mix, e.g. master-mix, reaction buffer, enzyme, surfactants, probe fluorophore/quencher, etc. or reagents and kits for the DNA extraction module	For the PCR-based module: change reagents to suitable/acquirable/practical reagents	Dynamic range, trueness, amplification efficiency, R^2 coefficient, RSD_r, LOQ (Annex II MPR[3)]) and specificity
	For the DNA extraction module: avoid hazardous chemicals and replace - phased-out reagents	DNA concentration, yield, structural integrity and purity of DNA extracts
Taxon-specific reference module targeting multiple copies in the genome	Replace the taxon-specific module with one targeting a single-copy reference gene	In case a method not fully validated is available, whose specificity is already demonstrated, conduct a method verification: dynamic range, trueness, amplification efficiency, R^2 coefficient, RSD_r, LOQ (Annex 2 MPR[3)]) In case a new method is developed, conduct an in-house validation according to MPR– Phase 1[3)]
Dynamic range does not comply with current MPR document	Adapt/extend dynamic range to current MPR criteria	Trueness, amplification efficiency, R^2 coefficient and RSD_r for non-compliant levels
Loss of specificity	Develop a new method	All performance parameters required by the MPR[3)]

Source: European Network of GMO Laboratories (ENGL)

5 Conclusions

These guidelines provide recommendations on how laboratories should assess and validate modifications to EU reference PCR methods for GMO detection and quantification, so that the methods remain functional and compliant to Annex III of Regulation (EU) No 503/2013[2)] and to the criteria listed in the ENGL guidance document 'Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing'.

Method adaptations may become necessary over time because of revised performance criteria, technological developments, practicability considerations or possible unavailability of key reagents. At the same time, any modification must be evaluated to ensure that the analytical performance of the method remains fit for purpose.

Validated methods should, in principle, be applied as originally validated, but limited procedural changes may be acceptable when supported by appropriate experimental verification. The extent of re-assessment depends on the type of modification introduced. In all cases, laboratories using adapted procedures remain responsible for demonstrating that the method, under their conditions of use, continues to meet the latest ENGL minimum performance requirements.

The document also highlights that not all method modifications have the same significance; changes to experimental conditions such as reaction volume, PCR annealing temperature, DNA extraction or PCR reagents may be manageable through targeted re-evaluation of performance parameters, e.g. dynamic range, trueness, amplification efficiency and repeatability. By contrast, when specificity is compromised due to changes in analytical conditions or to the emergence of a new analyte that cross-reacts with the method, a new method should be developed and validated, in case suitable alternatives are not available.

A further important conclusion concerns older methods that no longer fully align with the current MPR. In particular, methods previously validated with the ΔCq approach should be adapted to the two-standard-curves approach, and methods with non-compliant dynamic ranges should be reassessed and extended where necessary. Likewise, taxon-specific reference modules targeting multiple-copy genes should preferably be replaced by validated single-copy reference modules to improve the metrological quality and comparability of quantification.

From an operational perspective, the document supports pragmatic harmonisation in laboratories where this does not compromise performance. Examples include aligning annealing temperatures between GM and taxon-specific modules, reducing reaction volumes to 25 µL or less, and replacing phased-out reagents with suitable alternatives. Such changes may improve practicability, cost-efficiency and long-term usability, but only if supported by the recommended experimental checks.

Overall, laboratories and method developers should ensure that validated methods remain robust, practically applicable and aligned with the current regulatory framework, also when modifications to the original validation conditions become necessary. These guidelines therefore provide a proportionate, practical and detailed basis for conducting assessments of method performance parameters when modifications to the originally validate methods are introduced.

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List of abbreviations

Abbreviations	Definitions
Cq	Quantification cycle
CRM	Certified Reference Material
DNA	Deoxyribonucleic Acid
dPCR	Digital Polymerase Chain Reaction
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	Genetically Modified
GMO	Genetically Modified Organism
ISO	International Organization for Standardization
JRC	Joint Research Centre
LOD	Limit of Detection
LOQ	Limit of Quantification
MPR	Minimum Performance Requirements
µL	Microliter
NRL	National Reference Laboratory
R^2	Coefficient of Determination
PCR	Polymerase Chain Reaction
RSDr	Relative Standard Deviation of Repeatability
WG	Working Group

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