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Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing – part 2

European Network of GMO Laboratories

European Union Reference Laboratory for Genetically Modified Food and Feed

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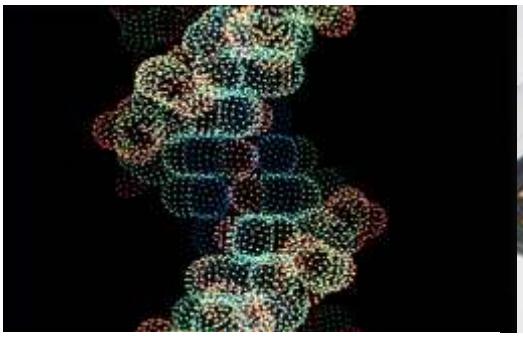

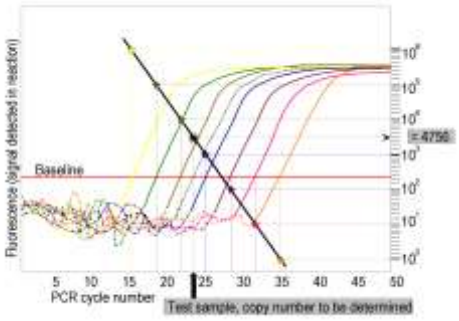

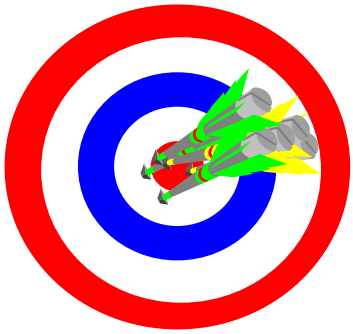

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Contents

Abstract

The document "Definition of Minimum Performance Requirements for analytical methods of GMO testing" (ENGL 2015) is a guidance developed by the ENGL and the EU Reference Laboratory for Genetically Modified Food and Feed (EURL GMFF). It supports the development and validation of methods for GMO analysis submitted in the frame of applications for EU market authorisation of GMO products, as well as those to be used in the official control of food and feed. The guidance document provides definitions and requirements to assess the performance of the methods developed for the detection and quantification of GMOs based on real-time PCR.

More recently, technologies like digital PCR were successfully applied in various fields for the quantification of nucleic acid molecules, including GMOs. Moreover, GM animals and products developed by means of so-called new genomic techniques (Broothaerts *et al*. 2021) are being released on the market and present additional challenges from the analytical viewpoint.

Therefore, ENGL experts have extended the original method performance parameters for guiding users in the development and validation of digital PCR methods for GMO analysis.

While the developments in the fields of GM animals and new genomic techniques are still evolving, in addition to the Minimum Performance Requirements guidelines also specific recommendations for methods for the detection and quantification of organisms with short genomic alterations and of GM animals are provided. Given that developments in the fields of GM animals and new genomic techniques are ongoing, these recommendations may be further elaborated in the future.

Foreword

The working group on Method Performance Requirements (WG-MPR) was established based on a mandate adopted at the 37th meeting of the Steering Committee of the European Network of GMO Laboratories (ENGL) on 18-19 June 2019. The WG has reviewed the current acceptance criteria and performance requirements for the validation of analytical methods for GMOs in view of new technology developments. A number of adaptations were made in order to extend the guidance document to methods based on digital PCR. The WG also established recommendations for the assessment and validation of methods aimed at detecting and quantifying GM animals and organisms obtained by new genomic techniques and derived products.

The working group was chaired by Francesco Gatto, European Commission Joint Research Centre (JRC). The members of the working group were: Christin-Kirsty Baillie, Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit (LGL) (DE) (until November 2020); Wim Broothaerts, Marco Mazzara and Christian Savini, European Commission (JRC); Malcolm Burns, LGC (UK); Frederic Debode and Aline Marien, Centre Wallon de Recherches Agronomiques (CRA-W) (BE); David Dobnik, National Institute of Biology (SI); Lelde Grantiņa-Ieviņa, BIOR (LV); Emilie Dagand, Lutz Grohmann and Kathrin Lieske, Federal Office of Consumer Protection and Food Safety (BVL) (DE); Ugo Marchesi and Daniela Verginelli, Veterinary Public Health Institute for Lazio and Toscana Regions (IT); Nina Papazova, Sciensano (BE); Jan Pieter van der Berg, Wageningen Food Safety Research (WFSR) (NL); Kamila Zdeňková, University of Chemistry and Technology (CZ).

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1 Introduction

The European Union Reference Laboratory for Genetically Modified Food and Feed (EURL GMFF) and the European Network of GMO Laboratories (ENGL) are tasked to define the performance parameters for methods of detection, identification and quantification of GMOs in the frame of the implementation and enforcement of related EU legislation.

These performance parameters are laid down in the guidance document "Definition of minimum performance requirements for analytical methods of GMO testing" (MPR) (ENGL 2015). This guidance is primarily intended for applicants seeking authorisation for GMO products in the EU (Regulation (EC) No 1829/2003), as well as for National Reference Laboratories (NRLs), or any other institution, wishing to develop methods for GMO analysis to be used in the context of official controls according to Regulation (EU) No 2017/625.

The MPR, in force since October 2015, applies to methods based on polymerase chain reaction (PCR), particularly to real-time PCR methods, for the detection, identification and, when applicable, quantification of GMOs, in combination with methods for DNA extraction.

In view of new scientific and technological developments, a need for the re-evaluation and potential expansion of the guidance document has been flagged. Whereas the current MPR document applies to real-time PCR methods, approaches using digital PCR have been recently shown to be widely applicable for the quantitative analysis of nucleic acids, including the quantification of GMOs (Pecoraro *et al.* 2019). The use of this novel technology for the enforcement of EU legislation required the ENGL to review and to amend the list of performance criteria for this technology.

In addition, new GMO products, including some derived from GM animals or others developed by using new genomic techniques (NGTs)([1]) (Broothaerts *et al.* 2021), are approaching the market for food or feed. Methods for the detection and quantification of such products may need further recommendations in respect to the performance parameters, as the current ones were mainly designed for the purpose of detecting and quantifying GM plants with recombinant DNA insertions.

The ENGL and the EURL GMFF have therefore established method performance requirements and acceptance criteria for the application of digital PCR methods for the detection, identification and quantification of GM food and feed. Moreover, recommendations are provided for the assessment of the performance parameters of PCR-based methods in view of their specific application for the detection and quantification of food and feed obtained by NGTs or derived from GM animals.

This document takes into account other relevant ENGL recommendations (Pecoraro *et al.* 2019; ENGL 2019) and ISO standards (ISO 2019a; 2019b).

1.1 Digital PCR

Digital PCR (dPCR) represents a measurement principle allowing the quantification of target nucleic-acid sequences. In particular, the reaction mixture is compartmentalised into hundreds or thousands of sub-samples (also called partitions), and each of these is analysed for the presence or absence of the target sequence. Thereby, the measurement result is based on an end-point analysis, i.e. a series of positive and negative outcomes, which can be considered as binary signals. Quantification of the target in the whole reaction mixture is obtained by converting the fractions of positive and negative partitions using binomial Poisson statistics. The latter approach requires the validity of a number of assumptions for the specific measurement (see Pecoraro *et al.* 2019).

Digital PCR provides accurate quantification and it is recognised to be less sensitive to PCR inhibitors and more suitable for multiplexing (Whale *et al.* 2020). In particular, dPCR multiplexing could improve the efficiency of the analysis, allowing a reduction of the amount of reagents and of the sample used, and at the same time improving the accuracy of quantification by determining the ratio between GM and taxon targets in the same reaction, thus reducing the variability in the reaction setup. Furthermore, dPCR is ideally suited to detect low target concentrations because of the high number of partitions.

([1]) NGTs are techniques that are capable of altering the genetic material of an organism and that have emerged or have been developed since 2001

Another advantage of dPCR is that it allows nucleic acid target quantification without reference to a calibration curve. This mitigates any potential matrix difference between the calibrant and the test samples, which could affect the accuracy of quantification. It is however recommended that reference materials are still included to check the accuracy of the method.

Users should consider that high target concentrations may saturate the capacity of the dPCR instrument and would invalidate the assumptions required for the data evaluation via Poisson statistics. Therefore, a prior estimation of the target copies concentration is required for some samples.

1.2 Food and feed products obtained by new genomic techniques

NGTs encompass a plethora of techniques that can generate different kinds of alterations in the genome, varying from single nucleotide variations (SNVs) to deletions and insertions of many base pairs (Broothaerts *et al.* 2021). Their application for agricultural and food purposes has been already reported in a wide range of organisms, including plants, animals and fungi (Parisi *et al.* 2021).

The ruling of the Court of Justice of the European Union in 2018 (CJEU 2018) established that the mutagenesis exemption in Directive 2001/18/EC only applies to techniques/methods of mutagenesis which have conventionally been used in a number of applications and have a long safety record. Therefore, under the current legal framework, organisms and derived products developed with NGTs, e.g. genome editing techniques, are considered GMOs that are subject to the same regulations as GMOs developed by recombinant DNA technologies.

Recently, a report released by the ENGL (ENGL, 2019) reviewed the possibilities and acknowledged the challenges for the detection of food and feed plant products obtained by NGTs, particularly for those products containing small alterations.

Currently, many of the organisms derived from genome editing are characterised by single and short nucleotide alterations (deletions/insertions)([2]). In some cases, multiple genetic alterations are generated such as modifications in all alleles of a gene or in different genes (Najera *et al.* 2019). NGTs may also be used together with a recombinant DNA donor template for the site-specific insertion of novel DNA sequences into the genome. This approach would create two unique junction sequences in the genome that can be targeted by PCR methods as for GMOs resulting from recombinant DNA techniques.

The focus of this guidance document is on organisms containing short genomic modifications that resemble the type of variations that could be potentially found in organisms developed by conventional breeding techniques.

The current state of knowledge indicates that it should be possible, in principle, to develop real-time PCR and digital PCR methods for the detection and quantification of short nucleotide alterations, including the alteration of a single nucleotide (Chhalliyil *et al.* 2020; Peng *et al.*, 2020; Zhang *et al.* 2021). However, fulfilling the specificity requirements for the event-specific detection, as it is set by the EU legislation for GMO analysis methods, might not be straightforward for such detection methods, if only a single nucleotide is altered (Weidner *et al.* 2022). It remains to be investigated whether it is possible to unambiguously identify the genome-edited organism on the basis of these short nucleotide variations and distinguish it from similar organisms that have resulted from conventional breeding (Grohmann *et al.* 2019; Broll *et al.* 2019; Ribarits *et al.* 2021; Shillito *et al.*, 2021). Notwithstanding this caveat, some recommendations are provided here for the development and validation of such methods.

1.3 Food and feed from GM animals

GM animals have been developed for various purposes such as human consumption, improving their rearing and for specific features (e.g., disease resilience, milk and meat composition, increased muscle growth), as companion animals (ornamental fluorescent fish, miniature animals), for the production of industrial or

([2]) European GMO Initiative for a Unified Database System - https://www.euginius.eu

pharmaceutical compounds, as public health tools (control of insects that transmit diseases) or for research studies (Lievens *et al.* 2015).

GM traits are generally obtained by means of genetic constructs combining promoters, coding sequences and terminators originating from other animal species. As these animal-derived sequences could show similarity or identity with DNA sequences from other animal species present in food and feed products, developing efficient screening methods based on these genetic elements may be challenging. One exception are the fluorescent characteristics brought to ornamental animals by the introduction of similar or identical genes (Debode *et al.* 2020). As mentioned above, genetic alterations in animals can also be obtained by NGTs, which may generate similar challenges for detection and quantification as mentioned earlier for NGT products (see section 1.2).

2 Performance requirements for digital PCR methods

This section describes how dPCR-based methods for GMO analysis should be assessed and validated. It complements and clarifies the ENGL guidance "Definition of minimum performance requirements for analytical methods of GMO testing" (MPR) (ENGL 2015) for this type of PCR methods.

The validation procedure is conceived in two distinct phases:

- Phase 1: information and validation data are evaluated to ascertain whether a method is suitable for a full validation (according to the method acceptance criteria):
- Phase 2: conduction of a full validation study to confirm the fitness for purpose of the method (according to the method performance requirements). The latter is usually assessed by a collaborative trial conducted according to international standards (ISO 1994) or guidelines (Horwitz 1995).

2.1 Method acceptance criteria (Phase 1)

This section provides a review of the method acceptance criteria for phase 1 as set by the MPR (ENGL 2015). These criteria have to be fulfilled before a dPCR method can be considered suitable for a collaborative validation study (*i.e.* for Phase 2).

The method should be assessed as fit for the intended purpose in line with relevant regulatory provisions (Annex III of Commission Implementing Regulation (EU) No 503/2013 or Annex I of Commission Regulation (EC) No 641/2004).

2.1.1 Acceptance criteria common to all modules of a method

The acceptance criteria set out for real-time PCR based methods (ENGL 2015) are also valid for dPCR methods with amendments for the acceptance criteria applicability and practicability.

Applicability: a dPCR method developed for GMO analysis should fulfil the same requirements as in section 2.1.1 in ENGL 2015.

An event-specific PCR method should be applicable to the various types of food and feed (the various matrices) that are expected to be found on the market, including highly processed food and feed products. It is therefore emphasised here that event- and the taxon-specific reference systems should amplify sequences of comparable length for accurate quantification (Debode *et al.* 2017). The amplicons should also not exceed a recommended length of 150 bp.

Note.

In duly justified cases the amplicon length could exceed that limit. Data should demonstrate that the method does not result in a bias of the relative quantification of the GMO when applied to fragmented DNA of average size corresponding to the amplicon one in comparison to intact DNA (Gryson, 2010).

Practicability: The acceptance criteria for real-time PCR are also applicable to dPCR-based methods (see section 2.1.2 in ENGL 2015) with modifications on limitations associated with the new type of apparatus and the total reaction volume. Digital PCR instruments may require the use of dedicated reagents, reducing the likelihood of transferring a method developed on one instrument to another without further optimisation. Therefore, a dPCR method is considered practicable, if the application to another dPCR instrument can be successfully demonstrated. Alternatively, the method developer may demonstrate that the method has been successfully transferred into a real-time PCR format.

The limitation of the total reaction volume applied to real-time PCR methods is not applicable to dPCR systems that make use of fixed volume partitions to compartmentalise the sample.

Some dPCR applications may include the use of restriction enzymes, for example, to decrease the viscosity, thus increasing the maximum possible amount of DNA loaded per reaction, to linearise circular DNA or to separate linked target copies. The method developer should provide the scientific justification for the use of restriction enzymes. In such cases, it is recommended that the restriction step for the test sample and the subsequent amplification occur as two consecutive steps in the same reaction tube, to maximise practicability of the protocol. In addition, the restriction enzyme should not cut within the target PCR amplicon and should be

insensitive to DNA methylation, to avoid incomplete DNA digestion. The use of random fragmentation techniques (*e.g.* sonication) is not acceptable because cleavage could occur within the target sequence in a non-reproducible manner and might negatively affect the sensitivity of the method.

2.1.2 Acceptance criteria applicable to DNA extraction modules

Similarly to real-time PCR-based methods, DNA extraction modules already validated by the EURL GMFF do not require further validation, provided that the DNA extraction module has been declared fit for the purpose for the same species and on the same type of matrix.

The criteria established by ENGL are amended as follows:

DNA concentration: see section 2.2.1 in ENGL 2015.

DNA yield: see section 2.2.2 in ENGL 2015.

DNA structural integrity (size and damage status): see section 2.2.3 in ENGL 2015.

Purity of DNA extracts: purity of DNA extracts for using dPCR methods should fulfil the same requirements as in section 2.2.4 in ENGL 2015.

This can be assessed by means of an inhibition test performed with a validated taxon-specific reference method in a real-time PCR format (see section 2.2.4 in ENGL 2015 or Annex 2 Hougs *et al.* 2017) or by dPCR as indicated below.

Digital PCR is commonly regarded as less sensitive to the presence of PCR inhibitors (Pecoraro *et al. 2019)*. However, it should be considered that different methods may show different levels of susceptibility to inhibition (Iwobi *et al.* 2016; Demeke and Dobnik 2018; Morcia *et al.* 2020).

In principle, dilution of the DNA sample reduces the concentration of potential inhibitors in the reaction. Therefore, the estimated concentration of the target across the serially diluted samples should be in line with the expected fold differences in the absence of PCR inhibitors.

In dPCR, the absence of PCR inhibitors could be assessed on at least two serial dilution levels, each level measured in at least duplicate. The average of the absolute copies per reaction measured in the diluted samples multiplied by the dilution factor should not differ by more than 25 % from the average of the absolute copies per reaction measured on the highest concentration (lowest dilution). A relative deviation below or equal to 25 % is generally recognised as a benchmark for the performance of methods for GMO testing (Lievens *et al.* 2016). The highest concentration level should correspond to at least the amount required for the subsequent PCR analysis.

The formula below can be used to evaluate the deviation due to the presence of PCR inhibitors:

$$\frac{|\overline{Cp_u} - \overline{Cp_d} \cdot d|}{\overline{Cp_u}} \leq 0.25$$

$\overline{Cp_u}$= Average of copies per reaction at the undiluted or highest concentration level

$\overline{Cp_d}$ = Average of copies per reaction at the diluted concentration level

d = dilution factor of the diluted concentration level

Table 1 provides an example of an inhibition test showing the absence of PCR inhibitors (difference, in copy numbers per reaction, between the diluted and undiluted samples relative to the undiluted sample, expressed as a percentage).

Alternative approaches can be found in ISO 20395 (ISO 2019a).

Table 1. Example of an inhibition test with a DNA extract from maize showing the absence of PCR inhibitors: taxon-specific reference system (*hmg*), dilution levels (initial concentration, 1:4, 1:16), each level tested in duplicate. Cp = copies; rxn = reaction.

Accepted partitions	Positive partitions	Cp/rxn	Average Cp/rxn	Dilution factor	Average Cp/rxn x Dilution factor	Relative difference (%)
13809	12415	62398	61085*	1	61085*	n.a.
17080	15181	59771				
15519	6688	15341	15196	4	60782	0.5%
17598	7476	15050				
17373	2525	4274	4313	16	69011	13%
18792	2778	4353				

* Reference value

Source: data simulate an inhibition test.

2.1.3 Acceptance criteria applicable to digital PCR modules

The performance parameters defined for real-time PCR modules are widely applicable to dPCR. For some of them, further clarification is provided for specific experimental settings.

It is recognised that parameters linked to the characterisation of the calibration curve as in real-time PCR are not generally applicable to dPCR.

Specificity: Requirements set in section 2.3.1 in ENGL 2015 are also applicable to dPCR methods.

In particular, a dPCR method should amplify a single target sequence and display a single cluster derived from positive partitions. The cluster derived from the positive partitions should be clearly separated from the cluster from the negative partitions. The number of intermediate fluorescence signals (also known as "rain") should be limited (see Annex 1). For a multiplex dPCR method, the criteria should be assessed for each individual target/fluorophore.

For taxon-specific modules, the target of taxon-specific modules should be unique per haploid genome copy. The absence of copy number variations across a globally representative collection of the taxon should be demonstrated. Recent studies have identified a list of suitable taxon-specific reference methods for plant species that could be used (Jacchia *et al.* 2018; Demeke and Eng 2018; Paternò *et al.* 2018).

A submission of a multiplex dPCR method should be accompanied by evidence from *in silico* and experimental analyses that the combined PCR modules are amplifying exclusively the target sequences (see Annex 4). An *in silico* analysis should demonstrate the absence of interactions among primers and probes from PCR modules and should exclude the appearance of unexpected amplification products by analysing all combinations of primers and probes. If unexpected amplification products arise, they have to be investigated further by experimental analysis. For further guidance see the ENGL report (Grohmann *et al.* 2021).

Note: unintended amplification products (i.e. from a non-perfect match) may usually amplify at a lower efficiency. Following completion of the thermal cycling run, this can result in an endpoint fluorescence that is lower than that for the actual target. Consequently, such amplifications are visible in the dPCR measurement as an additional (distinct) population of signals with fluorescence values between the negatives and the true positives. The presence of unintended clusters of signals should be avoided since it may complicate the analysis, affect the separation of positives from negatives, and ultimately lead to a misclassification of signals.

Dynamic Range: requirements set for the dynamic range for real-time PCR methods are also applicable to dPCR methods (see section 2.3.2 in ENGL 2015). Thus, the dynamic range should be assessed in terms of

trueness and precision, either on an absolute concentration level for the single PCR module or in relative concentration ratios for combined PCR modules (i.e. GM assay and taxon-specific assay).

For the combined PCR modules, the dynamic range should comprise at least 0.09 % and 4.5 % in GM mass fraction.

Note: the conversion copy number ratio to mass fraction should be performed considering the conversion factor determined by the EURL GMFF on the certified reference material (CRM) associated with the authorised GMO (EURL GMFF 2019; Corbisier & Emons 2019; Corbisier *et al.* 2022) or in the absence of this, on the zygosity factor, established on a positive control sample (see Annex 2).

Trueness: see section 2.3.3 in ENGL 2015.

Amplification Efficiency: The amplification efficiency derived from the standard curve is not applicable to dPCR methods based on end-point fluorescence reading.

R^2 coefficient: The R^2 coefficient derived from the standard curve is not applicable to dPCR methods based on end-point fluorescence reading.

Linearity:

Definition: Ability of a method of analysis to provide, within a specified range, an instrumental response or results directly proportional to the relative content of the GM- to the reference target.

Acceptance criteria: The degree of linearity within the dynamic range should be characterised in terms of slope and coefficient of determination (R^2) by regression analysis of the expected vs. observed GMO content. Samples used for the evaluation of linearity should be in line with the requirements for dynamic range and may be calibration materials or a dilution series of a test sample.

The slope of a plot of "observed vs. expected value" should be 1.00 ± 0.25. The coefficient of determination (R^2) should be greater or equal to 0.98.

Linearity should be demonstrated on a minimum of 3 runs considered individually. Each run should include at least 5 concentration levels each tested in 3 or more PCR replicates.

Note: some dPCR instruments may not permit the analysis of the number of samples recommended above for the linearity assessment within a single run. In such cases, the method developer may use an alternative appropriate experimental design, taking steps to ensure that the same number of concentration levels and the same number of replicates are tested.

Precision - Relative Repeatability Standard Deviation (RSD_r): see section 2.3.6 in ENGL 2015.

Limit of Quantification (LOQ): see section 2.3.7 in ENGL 2015. In case of multiplex dPCR methods, the method developer should assess the LOQ under asymmetric conditions (LOQ_{asym}). The LOQ_{asym} is determined by testing serial dilutions of the target in the presence of a high number of copies of the other target(s) (20,000 copies per reaction, if possible). For further guidance see the ENGL report (Grohmann *et al.* 2021). For duplex quantitative real-time PCR methods (i.e. GM assay and taxon-specific assay), the LOQ is determined in asymmetric conditions following the provisions for dynamic range at 2.3.2 of the MPR, 2015.

Limit of Detection (LOD): see section 2.3.8 in ENGL 2015. In case of multiplex dPCR methods, the method developer should assess the LOD under asymmetric conditions (LOD_{asym}). For further guidance see the ENGL report (Grohmann *et al.* 2021).

Robustness: requirements set for real-time PCR methods are also applicable to dPCR based methods (section 2.3.9 in ENGL 2015) with modifications on the factors that should be tested. In particular, the following factors have to be tested for a dPCR module:

- Temperature ramp rate associated with thermal cycler brand and model;
- final concentration of the master mix;
- primer concentrations;
- probe concentration;
- annealing temperature.

2.2 Method performance requirements (Phase 2)

Method performance requirements are the minimum performance criteria that the method should demonstrate upon completion of the full validation study carried out according to internationally accepted technical provisions.

Digital PCR methods should meet the same performance requirements as real-time PCR methods assessed through a collaborative validation study (see Chapter 3 in ENGL 2015).

Precision - Relative Reproducibility Standard Deviation (RSD_R): see section 3.1 in ENGL 2015.

Trueness: see section 3.2 in ENGL 2015.

False positive rate (type I error rate): see section 3.3 in ENGL 2015.

False negative rate (type II error rate): see section 3.4 in ENGL 2015.

Probability of Detection (POD): see section 3.5 in ENGL 2015.

2.3 Method verification criteria

This section defines the criteria that a dPCR method should meet when a pre-established fully validated method is applied to new GM events (e.g. stack GM events) within the scope of Regulation (EC) No 1829/2003.

The applicant should provide the information in line with the requirements set in Annex 2 of the MPR guidance (ENGL 2015).

With respect to real-time PCR methods, the assessment should be based on data demonstrating that the criteria set for the following parameters are fulfilled:

- Dynamic Range;
- Trueness;
- Repeatability Standard Deviation (RSD_r);
- Linearity;
- Limit of Quantification (LOQ).

3 Recommendations for PCR-based methods for food and feed products obtained by new genomic techniques

Methods submitted for the detection and quantification of food or feed products obtained by NGTs have to fulfil the requirements as set out by the MPR guidance for real-time PCR methods (ENGL 2015) or digital PCR methods (see section 2 of this document).

The following recommendations are provided as additional clarification for the assessment of the performance parameters for detection methods for food and feed products obtained by NGTs, particularly if the altered DNA sequence is limited to single or short nucleotide variations. This section provides a guidance, which could be subject to further elaboration and adaptation to reflect the evolving knowledge and legal status of NGT products.

It may be technically possible to detect and quantify specific DNA alterations even when these are only composed of a single or a few nucleotide changes. It is recognised that detection of the DNA alterations alone does not necessarily allow an unambiguous identification of the GMO event and/or specific product or to distinguish it from organisms of the same species resulting from conventional breeding. Methods detecting short DNA alterations, without necessarily identifying the GMO event, may however be used as first-line screening tools, detecting the induced mutation but potentially also similar natural mutations.

Applicability

As for any event-specific PCR method, the applicant should discuss the validity and limitations of the detection method for the various types of food and feed (the various matrices) that are expected to be placed on the market (see also applicability in section 2.1.1).

Food and feed products obtained from organisms modified by NGTs may present multiple genetic alterations. For instance, modifications may be introduced in different parts of a gene, in all alleles as well as in different genes. In case the alterations may segregate in subsequent generations, the detection method(s) would be required to target all distinct alterations in the genome.

Specificity

Also for products resulting from the application of a NGT, the event-specific method submitted under Regulation (EC) No 1829/2003 should allow the unequivocal detection, identification and quantification of the modified organism in order to fulfil the requirements stipulated in Annex III of Regulation (EU) No 503/2013 or Annex I of Commission Regulation (EC) No 641/2004).

The specificity of the method is assessed both by *in silico* and by experimental testing in accordance with the requirements set out for real-time PCR (see section 2.3.1 in ENGL 2015) or dPCR methods (see section 0).

The *in silico* specificity assessment based on similarity searches in representative nucleotide sequence databases (e.g. EMBL, GenBank, etc.) should demonstrate the theoretical capacity of the method to differentiate between the target sequence and similar sequences.

To detect possible cross-reactivity with non-target DNA, experimental specificity of a method should be assessed by testing the following types of samples:

- organisms with significant non-target similarities identified during the *in silico* specificity assessment of the primers, the probe or putative amplicons; these should be reported and further assessed by experimental testing, where relevant;
- a range of representative non-modified organisms and species that could be intermingled in food and feed;
- where applicable, samples from varieties or breeds (or weeds) showing the same trait and phenotype based on the alteration of the same gene;
- GM plants for which certified reference materials are available at the date of submission of the application for authorisation in the EU (in order to prove absence of cross-reactivity against any of the inserted transgenic constructs).

Cross reactivity was observed for a method targeting a SNV if assessed in the presence of a large amount of DNA from the conventional non-GM counterpart of the species (Weidner *et al.,* 2022). Therefore, to detect possible non-specific amplifications, methods targeting single or short nucleotide variations should be tested on non-modified samples of the species under investigation at the highest DNA amount per reaction according to the submitted method protocol.

Limit of detection (LOD)

PCR methods targeting sequence alterations having high similarity to the sequence of the conventional non-GM counterpart of the species may be hampered by competition for the oligonucleotides (Peng *et al.*, 2018; Zhang *et al.* 2021). Therefore, a low sensitivity of the method may be observed when a small amount of the target is tested in the concomitant presence of a high amount of the non-modified target.

For such methods, the LOD, in terms of copies per reaction, should be determined by testing the analyte target in the presence of the non-modified DNA of the corresponding species at the highest DNA amount according to the method protocol. As described in section 2.3.8 of the MPR (ENGL 2015), the number of replicates tested per amount or concentration under this condition should be 60, with the LOD set at the lowest concentration yielding at least 59 positive results (ENGL 2015).

The use of a different approach should be supported by statistical evidence ensuring that the level of confidence required is reached (CEN 2021).

Robustness

The robustness of a method measures its capacity to remain unaffected by small and deliberate deviations from the conditions described in the experimental procedure.

A PCR method targeting a sequence that differs by one or a few nucleotides from similar sequences relies on a limited nucleotide variation to provide the specificity of the method.

It is possible that slight variations in PCR conditions may lead to the onset of non-specific signals when such a method is applied in the presence of non-target nucleic acid sequences sharing a high level of similarity (Weidner *et al.* 2022).

A negative control should be included in robustness studies at the highest amount per reaction, according to the submitted method-protocol. This negative control sample should be chosen from the most likely non-target organisms that were identified during *in silico* specificity analysis and may be the corresponding unmodified isogenic line (if available) or another material.

The target DNA should be tested at an amount corresponding to 3 times the LOD_{abs} for qualitative PCR methods, or at the LOQ_{abs} for quantitative methods, respectively. This should be conducted in a background of highly similar genomic DNA from the same species at the highest amount per reaction according to the submitted method protocol.

4 Recommendations for PCR-based methods for GM animal testing

Methods submitted for the detection and quantification of food or feed produced from GM animals should fulfil the combined requirements as set out by the MPR for real-time PCR methods (ENGL 2015) and the current guidance document for digital PCR methods (see section 2 of this document).

The following notes or amendments complement or clarify the requirements for detection methods for GM animals.

Unit of measurement

The outcome of quantitative GMO measurements on food and feed produced from GM animals should be expressed in terms of a GM mass fraction. The conversion from copies to mass fraction should be performed considering the conversion factor determined by the EURL GMFF on the CRM associated with the authorised GMO, or in the absence of this, on the zygosity factor, established on a positive control sample.

Applicability

Food and feed produced from GM animals can be diverse, similarly to what is observed for GM plants. For the purpose of the assessment and validation of methods for the detection and quantification of GM animals, the method developer should test the method for DNA extraction and PCR detection on the portion or tissue of the GM animal representing the foreseen main application (e.g. muscles for meat).

The method developer should discuss the validity and possible limitations of the detection method for the various types of food and feed (the various matrices) that are expected to be placed on the market.

Specificity

The proof of the specificity of the event- and of the taxon-specific methods should be demonstrated both *in silico* and by experimental tests in line with the MPR acceptance criteria (ENGL 2015) and section 2.1.1.

As for GM plants, the *in silico* specificity analysis should be performed by similarity searches in appropriate databases (e.g. EMBL, GenBank, Patent, etc.), including known sequences of GMOs and relevant species for food and feed purposes, e.g. animals, plants, microorganisms, etc. When the results predict possible unexpected amplification products, those should be further investigated by experimental testing or confirmed by other techniques (e.g. sequencing).

In the assessment of the experimental specificity, it should be demonstrated that the method exclusively detects the target event (for the GM module) or species (for the reference target module).

In particular, taxon-specific methods should be specific for the species of the GM animal. However, it may be possible that some methods show cross-reactivity with closely related species. In these cases, the taxon-specific method should be evaluated on a case-by-case basis and the relevance of the detected non-target species should be assessed considering its relevance for food/feed control purposes (e.g. a simultaneous detection of domestic pig and wild boar could be considered acceptable due to the limited use of boar meat).

In some cases, methods targeting animal species-specific reference genes have generated non-specific amplification signals when tested with DNA from plant samples. This may happen when mitochondrial or ribosomal DNA targets are used (e.g. insects in Debode *et al.* 2017). Therefore, methods targeting animal species-specific genes should also be assessed on representative panels of DNA samples from plants commonly used for food and feed.

The experimental specificity of a method (incl. event-, element-, construct- and taxon-specificity) should be confirmed by testing samples from:

- the GM animal;
- the near-isogenic or parental non-modified organism;
- a representative set of samples from breed relatives of food/feed interest (e.g. to include wild boar for a GM pig);
- at least 10 non-GM animals from other species used in food or feed products (e.g. for a GM pig select other animals of food interest e.g. sheep, goat, cattle, chicken, turkey, etc.);

- if applicable, all GM animals for which reference materials are available at the date of submission of the application for authorisation in the EU;
- plant species relevant for food/feed purposes (e.g. maize, soybean, rapeseed, rice, wheat, etc.).

It is recommended to include in the specificity tests samples from those organisms that provided the elements used in the recombinant DNA constructs inserted into the GM animal. This is of particular interest for methods developed for screening and for which the donor organisms might be of food/feed interest.

Taxon-specific methods developed for quantitative purposes should target nuclear and single copy genes. Multi-copy genes are deemed acceptable only if there is no alternative single-copy gene available and if the number of copies is stable within the species. Mitochondrial gene targets are not suitable for quantification purposes due to their variable target copy numbers, except when the modification is inserted exclusively in the mitochondrial DNA. Only in this specific case, a mitochondrial gene target for the taxon-specific reference system would be preferable.

Note: lists of relevant animal species for experimental specificity analysis are available in Annex A of ISO 20813 (ISO 2019b).

Note: matrix or genomic DNA reference materials are suitable for specificity testing. Plasmid control samples are not suitable.

Note. Detection methods for bovine, ovine, porcine, chicken, goat, horse, donkey, turkey and goose DNA can be found in the ISO 20224 series of technical specification (ISO/TS 2020)

Dynamic Range

The dynamic range of the PCR method should be assessed according to the ENGL requirements.

For combined modules, the dynamic range should be assessed on the relative content of GM animal DNA in mass fraction. The portion or tissue of the GM animal representing the foreseen main application (e.g. muscles for meat) should be used as a reference.

The dynamic range of GM- and taxon-specific PCR modules should demonstrate a suitable level of trueness and precision in the range of absolute copy number levels established in the MPR (ENGL 2015).

Note: The absolute copy number can be estimated considering the genome size of various animals provided in Annex B of ISO 20813 or other relevant databases (Gregory, 2022).

5 Conclusions

The European Union Reference Laboratory for Genetically Modified Food and Feed (EURL GMFF) and the European Network of GMO Laboratories (ENGL) are tasked to define the performance parameters for methods of detection, identification and quantification of GMOs in the frame of the implementation and enforcement of related EU legislation.

The MPR, in force since October 2015, applies to methods based on polymerase chain reaction (PCR), particularly to real-time PCR methods, for the detection, identification and, when applicable, quantification of GMOs, in combination with methods for DNA extraction.

Digital PCR has been recently shown to be widely applicable for the quantitative analysis of nucleic acids, including the quantification of GMOs. The use of this novel technology for the enforcement of EU legislation required the ENGL to review and to amend the list of performance criteria for this technology.

In addition, new GMO products, including some derived from GM animals or others developed by using new genomic techniques (NGTs), are approaching the market for food or feed. Methods for the detection and quantification of such products need further recommendations in respect to the performance parameters, as the current ones were mainly designed for the purpose of detecting and quantifying GM plants with recombinant DNA insertions.

The ENGL and the EURL GMFF have therefore established method performance requirements and acceptance criteria for the application of digital PCR methods for the detection, identification and quantification of GM food and feed. Moreover, recommendations are provided for the assessment of the performance parameters of PCR-based methods in view of their specific application for the detection and quantification of food and feed obtained by NGTs or derived from GM animals.

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List of abbreviations and definitions

BLAST	Basic Local Alignment Search Tool
BLASTn	Nucleotide BLAST
bp	base pair
CRM	Certified Reference Material
dMIQE	minimum information for publication of quantitative digital PCR experiments
DNA	Deoxyribonucleic Acid
dPCR	digital PCR
CJEU	Court of Justice of the European Union
EMBL	The European Molecular Biology Laboratory
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
LOD	Limit of Detection
LOD_{abs}	Limit of detection in terms of copies per reaction
LOQ	Limit of Quantification
LOQ_{abs}	Limit of Quantification in terms of copies per reaction
MPR	ENGL guidance document "Definition of Minimum Performance Requirements for analytical methods of GMO testing" (2015)
NCBI	National Center for Biotechnology Information
NGT	New Genomic Techniques
NRL	National Reference Laboratory
PCR	Polymerase Chain Reaction
POD	Probability of Detection
R^2	coefficient of determination
Rs	resolution
RSD_r	Relative Repeatability Standard Deviation
RSD_R	Relative Reproducibility Standard Deviation
SNV	Single Nucleotide Variants
WG-MPR	Working Group on Method Performance Requirements
λ	Lambda, in digital PCR, the mean DNA target copies per partition

List of tables

Annexes

Annex 1. Recommendations for digital PCR methods

Statistical assumption and calculation

The outcome of a dPCR analysis is generally represented by the number of positive and negative partitions. The conversion of positive and negative rates into absolute copy number or the relative content of nucleic acid targets (ratio between GM and taxon-specific target copies) should be performed in line with ISO 20395 (ISO 2019a).

In measuring the signals converted afterwards into absolute copies per reaction of the target, the size of the partition may have a significant impact on the quantification of the targets present. At first, the partition volume has to be known for a correct calculation of the absolute copy numbers. Secondly, any variability between individual partition volumes may affect the accuracy of the results (Corbisier *et al.* 2015; Dagata *et al.* 2016; Pinheiro *et al.* 2012; Košir *et al.* 2017).

Unit of measurement

The relative GMO content should be expressed in terms of GM mass fraction (see Annex 2).

Method optimisation

Method optimisation can be performed by following the recommendations outlined in Pecoraro *et al.* 2019. An optimised dPCR method should display a clear discrimination between positive and negative signals.

It is recommended to use indicators of optimisation of the fluorescence signals to assess the separation between positive and negative clusters. A clear separation could be measured in terms of resolution (Rs) (Lievens *et al.* 2016). Rs above 2 should be observed when it is calculated as the difference in fluorescence between the peaks of positive and negative clusters, divided by the combined widths of the peaks. A suitable concentration of the target(s) should be used to assess the separation between positive and negative droplets (e.g. $\lambda = 0.7$ corresponds to a concentration in which approximately half of the partitions are expected to be positive). Further details for the analysis of intermediate fluorescence rate and resolution can be found in additional guidance documents (Pecoraro *et al.* 2019; Lievens *et al.* 2016).

In the absence of standardised tools, a picture of the fluorescence plot should document the separation between positive and negative clusters.

Quality criteria for reactions

A minimum number of partitions should be accepted for analysis according to the manufacturer's recommendation.

Intermediate fluorescence signals (i.e. rain) may be observed when suboptimal amplification occurs and may be due to various reasons, including co-purified PCR inhibitors or sub-optimal setup. Sub-optimal fluorescence may lead to misclassification of partitions generating a bias on the measurement result. Therefore, the rate of intermediate fluorescence should be limited. Scientific evidence has demonstrated that optimised droplet dPCR methods are capable of achieving a rate of intermediate fluorescence (i.e. 'rain') below 2.5 % (Lievens *et al.* 2016).

Qualification of reactions

Sporadic fluorescent partitions may appear in negative reactions. Although it is recognised that there is no clear consensus on the number of fluorescent partitions which qualify a sample as being not detected (§5.4 Pecoraro *et al.* 2019), it has been suggested that this could be set as a maximum of two. This value is provided for guidance only.

Annex 2. Determination of the zygosity ratio in the positive control sample

The so-called absolute amount of target DNA sequences is estimated in dPCR in terms of copy number per volume. The GM content relative to its ingredient (species DNA) is then expressed in terms of GM to reference-target copy number ratio.

However, the regulatory unit for the expression for analytical results of GMO quantification is GM mass fraction (P. Corbisier *et al.* 2017).

Therefore, when results are expressed first in terms of DNA copy number ratio, a conversion factor has to be used to convert the values into GM mass fractions. This conversion factor is established on the respective CRM according to the principle illustrated elsewhere (Corbisier and Emons 2019) and the established conversion factor can be found at https://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm.

In the absence of a CRM, such as in the case of a submission of a new application under Reg. (EC) No 1829/2003, the EURL GMFF can establish a zygosity factor on the positive control sample submitted, following the same dPCR measurement principles.

The zygosity ratio (GM-target to reference-target ratio) of the positive control sample used for the validation would be assessed by measuring the copy number of the GM-target and of the reference-target dPCR. Estimates of the zygosity ratio should be obtained on a sufficient number of test results, at least 15.

When an optimised protocol for dPCR has not been developed and validated, the amplification conditions of the optimised real-time PCR method are implemented for these purposes.

Annex 3. List of key experimental information that needs to be reported when using dPCR for GMO analysis, adapted from the dMIQE guidelines.

The update on the minimum information for publication of quantitative digital PCR experiments (dMIQE) has been published (Whale *et al.* 2020). These guidelines advocate that key experimental information associated with each dPCR experiment should be recorded in a harmonised fashion. The guidance supports that the use of such a harmonised reporting structure will aid in the design and replication of dPCR experiments, will provide critical information for scientific review inclusive of independent corroboration of conclusions, and will further promote and maximize the scientific impact and potential of this powerful and unique technology.

Whilst the published guidance has a broad remit and is generic across a number of dPCR applications, some of the pertinent information translates well directly into using dPCR for GMO analysis. The ENGL supports the rationale behind having a harmonised reporting structure for dPCR experiments in an effort to promote best measurement practice advice in this area. A recommended list of key experimental information to report when publishing dPCR for GMO analysis, adapted from the dMIQE, is presented here for consideration.

Analytical sample

The analytical sample taken for analysis, inclusive of volume/mass measurements and any handling/storage conditions and subsampling.

DNA extraction

Amount of the analytical sample used, and the DNA extraction protocol used, including any deviations from this. The number of extraction replicates and extraction blanks should also be provided.

DNA assessment and storage

The extracted DNA amount, concentration and volume, and how these were determined (e.g. spectrophotometric Nanodrop 2000). Where applicable, how the DNA quality and integrity was assessed. Storage conditions of the extract inclusive of information on buffer (pH), listing any derived aliquots and dilutions.

DNA modifications prior to dPCR

List any steps to further treat the DNA (e.g. use of restriction enzymes, sonication, bisulfite treatment, etc.), inclusive of additional dilution and purification stages.

dPCR method for GMO analysis

Cite the protocol used for GMO analysis, inclusive of target sequence and primer information (where possible) as well as any adaptations/changes from the method. For the dPCR exclusive aspect, describe any pre-extraction mixtures made, inclusive of components used and proportional volume of extract added. Other pertinent information to record includes oligo nucleotide concentrations, oligonucleotide manufacture, thermal cycling parameters and amplicon length.

Assay validation/verification

If it was necessary, provide information on any optimisation strategy used, inclusive of PCR components and thermal cycling parameters tested, pass and fail criteria, control materials used and assessment of any analytical specificity and sensitivity.

Data analysis

Detailed information related to the experimental design should be provided, inclusive of, but not restricted to:

- Partition classification method (thresholding)
- Number of valid partitions measured (including average and standard deviation)
- Number of positive and negative partitions
- Minimum and maximum number of droplets generated (droplet dPCR)
- Partition volume
- Copies per partition (λ or equivalent) (average and standard deviation)
- Technical level of replication
- Source and use of positive and negative controls (application for quality control or to estimate error)
- Example experimental results from positive and negative controls – ideally augmented through the use of scatter plots where applicable
- Precision (repeatability and reproducibility) estimates

If not included in the previous section on DNA modifications prior to PCR, provide details of the volume of the reaction, volume of template per reaction and the dilution factor of the template.

Details of the dPCR analysis program (source and version) should also be provided, as well as any statistical packages/methods used.

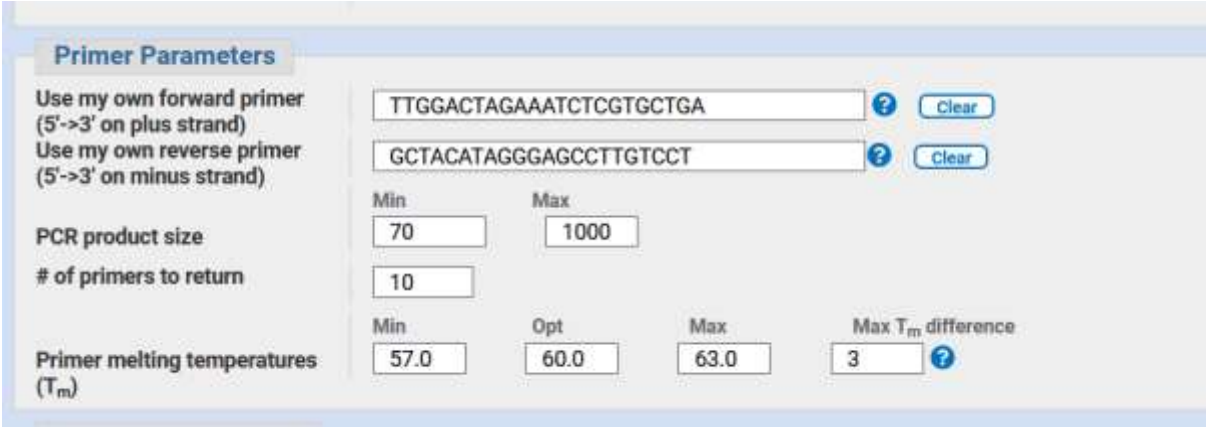
Annex 4. *In silico* specificity analysis

An *in silico* specificity analysis of PCR methods is important for predicting amplifications that may occur under the experimental conditions. Various approaches and settings could be used to perform this analysis.

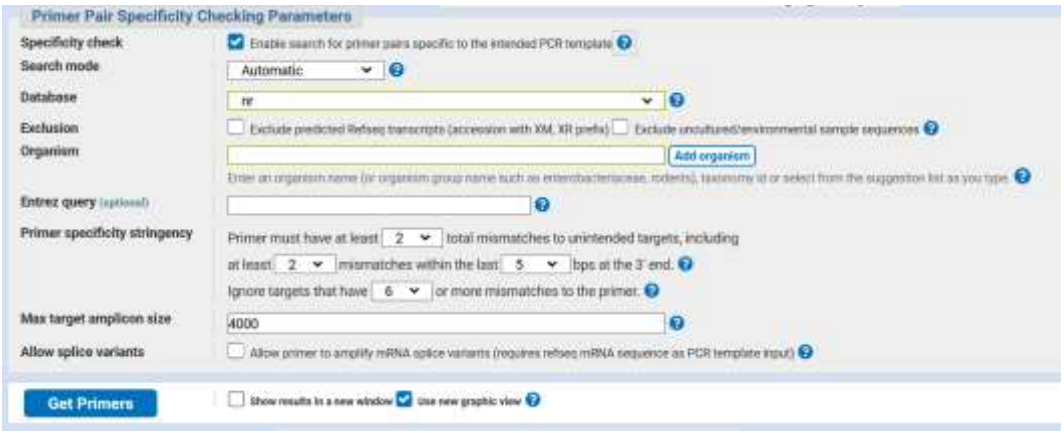
This Annex is illustrating how to perform an *in silico* analysis using NCBI[3] PrimerBLAST (Ye *et al.* 2012). This approach can be equally applied to PCR methods targeting GMOs derived from recombinant DNA techniques or new genomic techniques.

The primer sequences from the quantitative PCR method detecting the maize high-mobility-group gene (*hmg*) are used as an example for this analysis (ref. QT-TAX-ZM-002 in the GMOMETHODS database[4]).

1. Open the web page of the NCBI Primer-Blast at https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi;
2. In the section "Primer Parameters" insert the sequences of the primers. Use default settings for other fields.

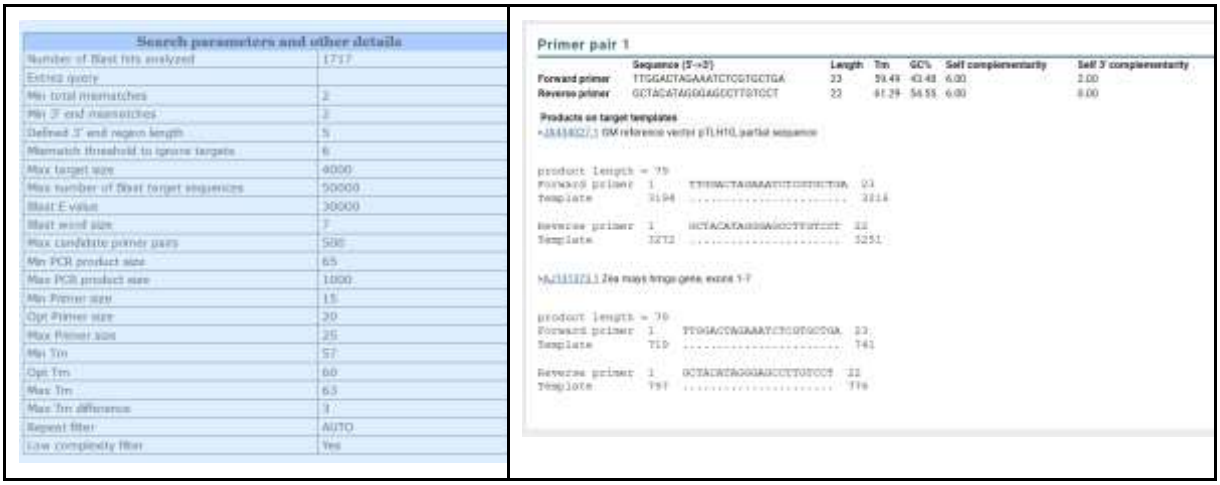


3. In the section "Primer Pair Specificity Checking Parameters" select "nr" as "Database" and delete "*Homo sapiens*" from the field "Organism".
4. Then Click the button "Get Primers".



[3] National Center for Biotechnology Information.
[4] GMOMETHODS database: https://gmo-crl.jrc.ec.europa.eu/gmomethods/

5. Results are generated as below.



Search parameters and other details	
Number of Blast hits analyzed	1717
Entrez query	
Min total mismatches	2
Min 3' end mismatches	2
Defined 3' end region length	5
Mismatch threshold to ignore targets	6
Max target size	4000
Max number of Blast target sequences	50000
Blast E-value	30000
Blast word size	7
Max candidate primer pairs	500
Min PCR product size	65
Max PCR product size	1000
Min Primer size	15
Opt Primer size	20
Max Primer size	25
Min Tm	57
Opt Tm	60
Max Tm	63
Max Tm difference	3
Repeat filter	AUTO
Low complexity filter	Yes

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TTGGACTAGAAATCTCGTGCTGA	23	59.49	43.48	6.00	2.00
Reverse primer	GCTACATAGGGIAGCCTTGTGCT	22	61.29	54.55	6.00	0.00

Products on target templates

>JX416027.1 GM reference vector pTLH10, partial sequence

```
product length = 75
Forward primer  1     TTGGACTAGAAATCTCGTGCTGA  23
Template        3194  ........................  3216

Reverse primer  1     GCTACATAGGGIAGCCTTGTGCT  22
Template        3272  ........................  3251
```

>AJ311971.1 Zea mays hmgb gene, exons 1-7

```
product length = 76
Forward primer  1    TTGGACTAGAAATCTCGTGCTGA  23
Template        715  ........................  741

Reverse primer  1    GCTACATAGGGIAGCCTTGTGCT  22
Template        797  ........................  776
```

The retrieved sequences showing significant similarity to both of the primers should be analysed for their origin (species) and possible alignment of the intervening sequence to the corresponding probe. In the example one sequence corresponds to the target (hmg from *Zea mays*) while the other refers to a plasmid developed for GMO analysis.

A BLASTn analysis using the amplicon sequence could also be informative. In this approach all the alignments that include both primer regions (potentially with no or a few mismatches) should be checked for their origin and for their further alignment to the probe sequence. However, in contrast to using PrimerBLAST, potential amplicons with a different length than the target amplicon may not be identified by using this approach.

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