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Guidance document on multiplex real-time PCR methods

European Network of GMO Laboratories
Grohmann L, Barbante A, Eriksson R, Gatto F, Georgieva T, Huber I, Hulin J, Köppel R, Marchesi U, Marmin L, Mazzara M, Narendja F, Owen H, Perri E, Scholtens I, Sovová T, Sowa S, Stebih D, Weidner C, Zdeňková K.

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Contact information
ENGL Secretariat
European Commission -Joint Research Centre (JRC)
Food & Feed Compliance Unit (F.5)
Via Enrico Fermi 2749. TP 201
I-21027 Ispra (VA) Italy
E-mail: JRC-ENGL-SECRETARIAT@ec.europa.e

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

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Foreword

The ENGL Working Group (WG) on multiplex PCR was established based on a mandate adopted at the 33rd meeting of the ENGL (European Network of GMO Laboratories) Steering Committee on 20-21 June 2017. The WG reviewed the different aspects concerning the application of multiplex PCR, identified needs for guidance for testing laboratories and described approaches to address them.

The working group has been chaired by Lutz Grohmann, Federal Office of Consumer Protection and Food Safety (BVL) (DE), and the members of the working group were (in alphabetical order): Alessandra Barbante, Council for Agricultural Research and Economics, Research Centre for Plant Protection and Certification (CREA DC) (IT); Ronnie Eriksson, National Food Agency (SE); Francesco Gatto, European Commission's Joint Research Centre; Tzveta Georgieva, National Center of Public Health and Analyses (BG); Ingrid Huber, Bavarian Health and Food Safety Authority (LGL) (DE); Julie Hulin, Centre Wallon de Recherches agronomique (CRA-W) (BE); Rene Koeppel, Official Food Control Authority Canton Zürich (CH); Ugo Marchesi, Veterinary Public Health Institute for Lazio and Toscana Regions (IT); Lucas Marmin, Service Commun des Laboratoires (FR); Marco Mazzara, European Commission's Joint Research Centre; Frank Narendja, Environment Agency Austria (AT); Heather Owen, Science and Advice for Scottish Agriculture (SASA) (UK); Elena Perri, Council for Agricultural Research and Economics, Research Centre for Plant Protection and Certification (CREA DC) (IT); Ingrid Scholtens, Wageningen Food Safety Research (WFSR) (NL); Tereza Sovová, Crop Research Institute (CZ); Sławomir Sowa, Plant breeding and Acclimatization Institute National Research Institute (PL); Dejan Štebih, National Institute of Biology (SI); Christopher Weidner, Federal Office of Consumer Protection and Food Safety (BVL) (DE); Kamila Zdeňková, University of Chemistry and Technology (CZ).

Executive Summary

Polymerase Chain Reaction (PCR) is considered the gold standard technique for the detection and quantification of Genetically Modified (GM) food, feed and seeds. The combination of two or more PCR methods in a single reaction tube is referred to as multiplex PCR and is a powerful approach already adopted by many testing laboratories. Multiplex PCR can address the need for increased and more efficient testing for the presence of GMOs in food, feed or seeds and for the quantification of GM events.

Although real-time PCR has been used for GMO testing for many years as well as for research, forensic or diagnostic purposes, a comprehensive guidance on the development and implementation of multiplex PCR is currently not available.

This document has been developed by experts from the European Network of GMO Laboratories (ENGL). It provides background information on the technical aspects of multiplex PCR (mpPCR), including information on instruments, fluorescent dye selection and reaction controls. It gives guidance on how to implement a previously validated mpPCR method (i.e. method verification), as well as how to develop and validate a new mpPCR method.

Guidance on criteria for the acceptance of methods, examples of how to assess these criteria and troubleshooting tips are given. Finally, a list of currently used mpPCR methods is included.

1 Scope

This document provides guidance for the development of new qualitative and quantitative multiplex real-time methods (mpPCR) and their implementation in a routine testing laboratory for food, feed and seeds. When implementing a mpPCR method, the laboratory should perform either an (in-house) validation or a verification of the method before its use.

The various method performance parameters of the specific requirements for mpPCR are described and examples are given. Several different aspects have been considered, including the selection of the fluorophores, the master mix and the real-time PCR instrument used. The technical aspects are examined and recommendations are made on the choice of the detection and quantification strategy. Finally, a separate chapter is dedicated to troubleshooting (e.g. underperforming method, no or poor signal strength, cross-talk).

In addition, a collection of existing mpPCR methods with emphasis on GMO detection is presented.

2 Introduction

Due to their high sensitivity and specificity, Polymerase Chain Reaction (PCR) based methods for the detection of nucleic acids are used in many different areas, including testing of food, feed and seeds. The common PCR assay is using a single primer pair (singleplex) to amplify one target sequence. By adding for example a second primer pair (duplex), or multiple primer sets (multiplex), the parallel amplification of different target sequences in one reaction is possible. The main advantages of multiplex real-time PCR (mpPCR) approaches are speed and cost-efficiency, which could significantly facilitate the screening process for food, feed and seed testing. A higher testing throughput, lower pipetting errors and lower needed DNA amounts are further benefits. In addition, the validation process covers all applied primer sets at once and makes also the documentation more straightforward. Performing multiple PCR tests in a single tube has also the benefit to reduce consumables and reagents allowing a reduction of the environmental impact of the analysis.

Therefore, mpPCR assays have been used in an increasing number of applications, including the detection and quantification of DNA from GMOs, food allergens and species identification to uncover cases of mislabelling and food fraud (Ghovvati *et al*. 2009; Waiblinger *et al.* 2017; Köppel *et al.* 2021; 2020; 2019; Köppel, Zimmerli, and Breitenmoser 2010; Holst-Jensen *et al.* 2012; Datukishvili *et al.* 2015; Wang *et al.* 2018).

However, some drawbacks to this approach include a longer development time, possible incompatibility of primers and probes and multiplex-specific problems, such as competition between the amplification modules.

In principle, all available PCR formats can be multiplexed. In conventional end-point PCR, multiple amplicons of different lengths are visualized by staining of PCR products. In real-time PCR and digital PCR (dPCR) the detection of amplified DNA is based on the monitoring of fluorescence signals emitted e.g. by the degradation of hybridization probes labelled with spectrally distinct fluorophores (Fig. 1).

Most quantitative duplex PCR methods (real-time PCR or dPCR) use two different reporter fluorescent dyes attached to the probes (see paragraph 3.2.2) and can be applied for the simultaneous amplification of a species-specific gene (taxon-specific target) and the junction region of a particular GM event for relative quantification (Samson, Gullì, and Marmiroli 2010; Foti *et al.* 2006).

Multiplex methods based on conventional end-point PCR have the disadvantage that the difference in amplicon lengths among the targets may result in different PCR efficiencies and, consequently, the competition among PCR modules may affect the limits of detection. In comparison, mpPCR using fluorescence-labelled probes can qualitatively and quantitatively detect several different target sequences of similar lengths without such effects on PCR efficiencies and are thus superior to end-point PCR (Wittwer *et al.* 2001).

Due to the higher specificity (by using the probe) and sensitivity of real-time PCR as well as its broader applicability including quantification, this technique is the most frequently used and is currently the standard approach employed in the European GMO testing area (Ciabatti *et al.* 2017). This guidance document will therefore cover only the application of real-time PCR based multiplex methods. Recommendations for the application of multiplex dPCR are provided in a separate comprehensive ENGL document (Pecoraro *et al.* 2019).

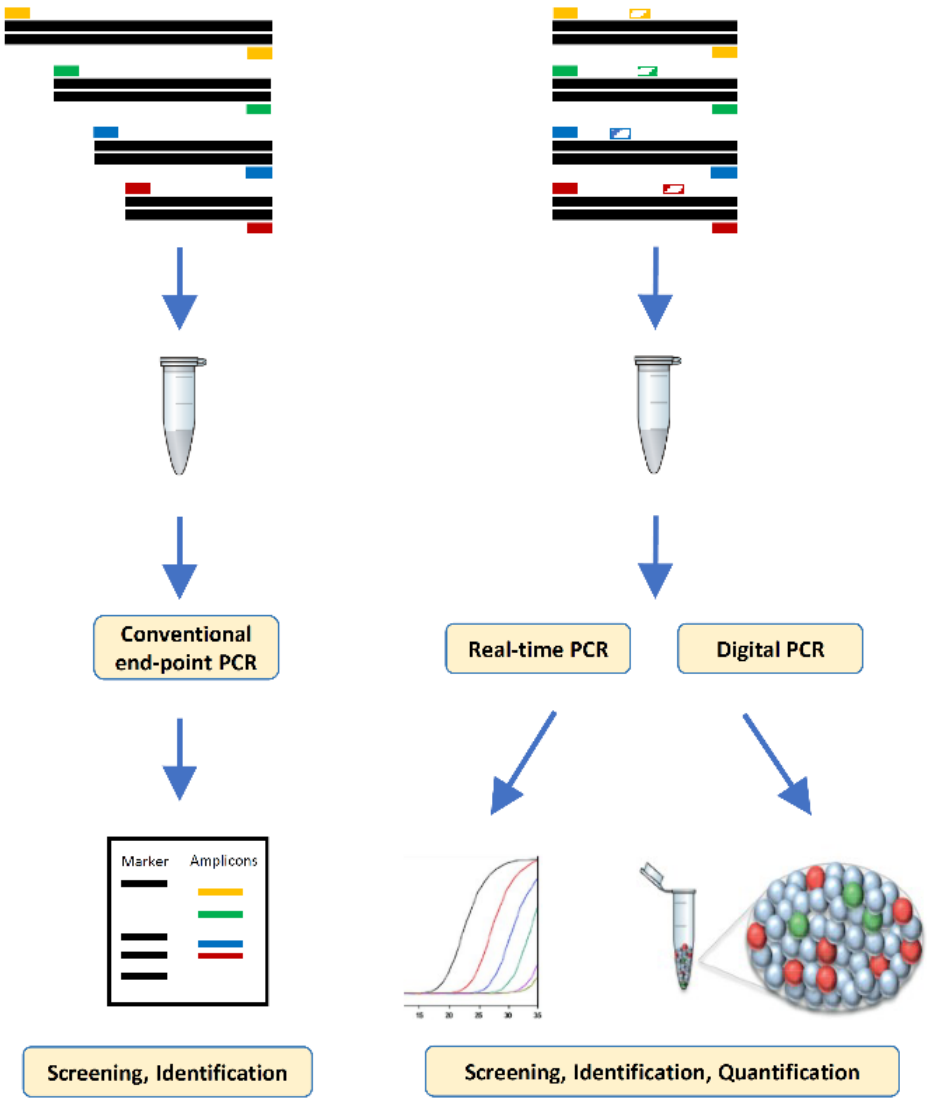

Figure 1 Schematic illustration of different approaches for mpPCR. The multiplex conventional end-point PCR method is based on the detection of amplicons of different size in horizontal or capillary electrophoresis (left). Multiplex real-time PCR or dPCR methods are based on the separate detection or quantification of the fluorescence from probes labelled with different fluorescent reporter dyes (right).

3 Technical aspects and detection strategies

3.1 General

Many different instruments with various thermal cycling and fluorescence detection systems are available. The type and number of fluorescence detection channels vary, as there is a range of different fluorophores available for probe labelling. Differences in the precision, with which these instruments can measure the fluorescence and the ability to discriminate signals from different labels, are important performance characteristics and require careful consideration. The fluorescence intensity and spectra of different dyes, as well as the noise in the fluorescence measurements, all affect the performance of probe-based real-time PCR applications. There are many different fluorophores available on the market. Their availability and specification, including their possible combination with quenchers, are important parameters for the design of a mpPCR method. The chosen reporter dyes have to be compatible with the instrument specifications and detectable emission wavelengths. These dyes should possess appropriate excitation wavelengths with little or no overlap in their emission spectra, which may cause bleed-through (cross-talk) of the fluorescence emission between the detector channels.

Optimisation of the conditions for a mpPCR method can be simplified by using specifically developed PCR master mix solutions. This usually results in reduced efforts for method optimisation.

In mpPCR the use of different fluorophores attached to the various hydrolysis probes allows the simultaneous detection and discrimination of the amplified DNA sequences (Höhne *et al.*, 2002; Waiblinger *et al.*, 2008). The number of targets is limited by the number of channels available in the PCR instrument.

Another detection strategy uses one (or two) fluorophore(s) for labelling of the different probes. Usually, this strategy may apply event-specific modules to screen those GMO which may not be detected by element- or construct-specific screening assays (Grohmann *et al.* 2017; Bahrdt *et al.* 2010). Such assays could confirm the absence/presence of the target sequences but, in case that an amplification signal is generated, they cannot provide an indication which of the targets was detected. This approach can be very useful for screening of uncommon promoters and terminators or the identification of rarely occurring GM events. In the case of a positive PCR result, separate singleplex PCR analyses of the sample DNA are required as the next step in confirming which specific target has been amplified.

3.2 Instruments, reporter fluorophores, quenchers and PCR master mix

For the application of a mpPCR method, numerous instruments, reporter fluorophores and quenchers as well as modifications of the probes are available.

The instrument and the degree of multiplexing (duplex, triplex, quadruplex etc.) are determining the reporter fluorophores to be used. Quenchers, in turn, should be selected to be compatible with the fluorophores. Depending on the modification of the hydrolysis probes (double quenched probes, minor groove binder, and locked nucleic acid probes…), the availability may be limited to certain fluorophore-quencher combinations.

3.2.1 Instruments

Most real-time PCR instruments used today are capable of multiplexing for the most common fluorophores. However, before implementing a mpPCR method the following general characteristics of the instrument should be checked:

- wavelengths and number of available detection channels,
- use of a passive reference dye required,

- instrument calibration for the selected fluorophores (e.g. custom dye calibration is needed),
- special requirements of the real-time PCR assay (e.g. cycling, ramp rate),
- software options for the analysis of data - manual setting of thresholds, baseline corrections, colour compensation.

This has an impact on which reporter dyes would perform best on a specific instrument. Some instruments use a reporter dye to correct the background in each well (i.e. passive reference dye). In this case, the corresponding channel is not available for detection of a probe. Therefore, one of the first steps in designing a mpPCR assay is the selection of reporter dyes appropriate for the detection channels of the instrument.

3.2.2 Reporter fluorophores

In most multiplex real-time PCR assays using hydrolysis probes, each target is identified by a signal emitted by a fluorophore serving as a reporter. The fluorophores have different excitation and emission wavelengths. Choosing a reporter dye for a multiplex PCR method requires the selection of those fluorophores with the least spectral overlap and with the most separated emission spectra. This will minimise the potential cross-talk, which can be caused by the bleeding of the fluorescent signal from one reporter to the adjacent channel of another reporter. In these cases, cross-talk may lead to artefacts giving false positive amplification signals and/or impair quantification.

Reporter dye combinations are instrument-specific and recommendations for combining the channels depending on the degree of multiplexing are available from the respective manufacturers. In duplex assays the FAM/HEX combination is currently one of the most used combinations.

The most commonly used reporter dyes that can be combined in a multiplex assay are shown in Table 1. Over the years, numerous new fluorophores have been developed for the use as reporters that are better adapted to the respective optical properties of the instruments, with narrower emission wavelength ranges to prevent cross-talk, or a higher emission intensity. Moreover, the assortment of dyes may differ between probe manufacturers and indications for suitable combinations of fluorophores used as reporter dyes. Recommendations for a specific instrument are in some cases provided by the manufacturers.

In addition, the selection of available dyes may be limited when modifying the classic hydrolysis probe to achieve a better specificity or sensitivity (quenching efficiency). For example, in double quenched probes, minor groove binder or locked nucleic acid probes, only certain dyes are available.

Lastly, the total fluorescence intensity should also be considered. The dye brightness is proportional to the product between the extinction coefficient (light energy absorbed) and the quantum yield (ratio of emitted photon over absorbed photons). These values depend on the experimental environment (solvent, salinity, pH etc.). Standard values can be retrieved from manufacturers or scientific papers (Invitrogen, 2010). In order to reduce the possibility of cross-talk, it is preferable to use a bright dye such as 6-FAM for low copy targets (e.g. GM-specific targets), and a less-bright dye such as Cy5 or Cy3 for high copy targets (e.g. taxon-specific target).

Table 1 Examples of common reporter fluorophores for real-time PCR

Reporter Fluorophores	Maximum Excitation Wavelength (nm)	Maximum Emission Wavelength (nm)	Extinction coefficient ($M^{-1}cm^{-1}$)	Brightness
Atto425[a]	439	485	45,000	Medium
6-FAM[b,c]	492	516	83,000	High
TET[b,c]	521	542	73,000	High
VIC[b]	525	543	*n.a.**	*n.a.*
JOE[b]	520	545	75,000	*n.a.*
Yakima Yellow[c]	530	549	83,800	High
HEX[b,c]	533	559	73,000	Medium
Cy3[b]	554	568	150,000	Low
TAMRA[c]	552	578	90,000	High
ROX[b]	578	602	82,000	*n.a.*
Texas Red[b]	585	602	116,000	Medium
Cy5[b]	650	669	250,000	Medium
Quasar 705[c,d]	690	705	206,000	*n.a.*

[a]www.atto-tec.com; [b]www.aatbio.com/spectrum; [c]www.glenresearch.com; [4]www.biosearchtech.com/support/education/fluorophores-and-quenchers/quasar-dyes; n.a.=values for extinction coefficient or quantum yield in aqueous solutions are not available

3.2.3 Quenchers

When choosing quenchers for a multiplex assay it is highly recommended to use non-fluorescent quenchers also called dark quenchers. These quenchers absorb broadly and do not emit any fluorescence by themselves, thus minimising the risks of cross-talk effects, reducing the background fluorescence, and ensuring the compatibility with different instruments. The selection of the optimal quencher depends on the selected reporter dye. Table 2 provides a short overview on the most commonly used dark quenchers in dependence of the emission wavelength of the fluorophore. In general, the best combination between reporter dyes and quenchers is also available from the respective manufacturers.

Double-quenched probes[1] are available on the market with the particular advantage of a low background fluorescence. These probes have two quencher dyes, one at the end and the other closer to the reporter dye. The shortened distance between the reporter dye and the internal quencher quenches more thoroughly, enhancing signal sensitivity.

[1] https://eu.idtdna.com/pages/education/decoded/article/modification-highlight-zen-internal-quencher

Table 2 Examples of dark quenchers in relation to suitable emission wavelengths of the fluorophores.

Abbreviation	**Name**	**Emission wavelengths of the reporter fluorophore (nm)**	**Suggested Reporters (Examples)**
Dabcyl[a,c]	nonfluorescent quencher	380-530 (max 453)	Cyan500, FAM, TET, JOE, HEX
DDQ-1[c,d]	Deep Dark Quencher 1	400-530 (max 430)	ATTO-425, FAM
BHQ-0[a]	BlackHole Dark Quencher 0	430-520 (max 495)	AMCA, FAM
BHQ-1[a,d]	BlackHole Dark Quencher 1	500-580 (max 534)	FAM, TET, VIC, Yakima Yellow, JOE, HEX
BHQ-2[a,d]	BlackHole Dark Quencher 2	550-650 (max 579)	ROX, Texas Red
BHQ-3[a,d]	Black Hole Dark Quencher 3	620-730 (max 680)	ROX, Texas Red, Cy5, Quasar705
BMN-Q460[c]	nonfluorescent quencher	400-530 (max 460)	Atto 390, Atto 425, Atto 465
BMN-Q535[c]	nonfluorescent quencher	480-580 (max 535)	HEX, Yakima Yellow, CY3,
BMN-Q590[c]	nonfluorescent quencher	550-650 (max 590)	ROX, CY3.5, Texas Red
BMN-Q620[c]	nonfluorescent quencher	480-720 (max 620)	ROX, CY3.5, Texas Red
BMN-Q650[c]	nonfluorescent quencher	550-720 (max 650)	Cy5, Cy5.5
IB FQ[b]	ZEN/Iowa Black FQ 3’ quencher Iowa Black FQ and internal ZEN Quencher for double-quenched probes (ZEN/3’IBFQ)	420-620 (max 531)	FAM, ATTO488, TET, Yakima Yellow, HEX, JOE, ATTO532
IB RQ[b]	Iowa Black RQ	500-700 (max 656)	CY3, ATTO550, TAT565, ROX, Texas Red, Cy5
ZEN[b]	Internal Quencher for ZEN/Iowa Black FQ 3’ quencher Iowa Black FQ and internal ZEN Quencher for double-quenched probes (ZEN/3’IBFQ)	480-560	FAM, TET, VIC, Yakima Yellow, JOE, HEX

Abbreviation	Name	Emission wavelengths of the reporter fluorophore (nm)	Suggested Reporters (Examples)
BBQ[a,e]	BlackBerry quencher	550-750 (max 650)	AMCA, Cyan500, FAM, HEX, ROX, LC640, Cy5, Cy5.5
QSY[f]	TaqMan QSY nonfluorescent quencher	500-620	ABY, FAM, JUN, VIC
[a]www.tibmolbiol.de;[b]eu.idtdna.com;[c]www.biomers.net;[d]www.eurogentec.com; [e]www.biosyn.com; [f]www.thermofisher.com			

3.2.4 PCR master mix for multiplexing

A multitude of different real-time PCR master mixes is available. The choice has considerable impact on the development and performance of the mpPCR method.

Besides general considerations for the real-time PCR setup, the master mix should be compatible with multiplex applications. For example, some are formulated with increased concentrations of the different reagent components to ensure simultaneous multiple target amplifications. The performance of different master mixes can be compared experimentally (Woll *et al.* 2013; Köppel and Zimmerli 2010).

4 Reaction set up and use of controls

When performing mpPCR, particular care should be devoted to the preparation of the control solutions and the reaction mixtures.

4.1 Reagents and oligonucleotides

In mpPCR, the use of several DNA control solutions and oligonucleotides sets increases the risk of pipetting and dilution mistakes.

For a multiplex assay, all the primers and probes may be mixed in a unique stock solution to reduce the preparation time. It may be preferable to prepare a stock solution for each PCR module, so that it is easier to remove a PCR module from the multiplex assay, if needed.

4.2 Reaction controls

The choice of the controls should follow existing standards (ISO 24276, ISO 21570, ISO 21569). Accordingly, a mpPCR run should include at least:

- a positive DNA target control, which demonstrates the ability of the PCR run to detect a target DNA. The use of a positive control with a low target copy number is recommended to ensure proper sensitivity;
- an amplification reagent control (also called no-template control - NTC), which demonstrates the absence of nucleic acid contamination in the reagents used. A sample which does not contain the target template (e.g. extraction blank) may replace the NTC;
- a control for a quantitative mpPCR method, which demonstrates the reliability of the quantification result. This control may be a certified reference material or any other reference material with a known analyte content.

For a multiplex assay, the results from controls should be evaluated for each individual DNA target.
Depending on the controls, the use of stock solutions containing a mix of different controls may be preferred to reduce the preparation time (Annex A: DNA control stock solutions).

4.2.1 Single-colour multiplex PCR

In the case of single-colour mpPCR, the same fluorescence signal is obtained for all the targets. Therefore, it is necessary to demonstrate the proper amplification of each individual target.
A positive DNA target control should be used for each individual target, in separate wells.

4.2.2 Multi-colour multiplex PCR

In the case of multi-colour mpPCR, a different signal is obtained for each target. Therefore, a mixture of different targets may be used instead of independent positive DNA target controls. This would reduce the total number of wells used for controls.

For qualitative analysis, a mixture of all the targets may be used. The use of a low copy number for each target is preferred in order to ensure sensitivity.

For a quantitative duplex PCR, such as for taxon-specific and GM specific targets, the standard curve and the internal quality control should contain both targets in order to achieve similar amplification conditions with samples and controls.

A synthetic construct containing several target sequences may be used as control material for qualitative analysis. However, using such a control for quantitative analysis would require confirmation that the targets cloned one close to the other do not generate non-intended amplification products and behave in the analytical procedure sufficiently similar as genomic DNA. Separation of the targets may be obtained by linearization of the plasmid using restriction enzymes targeting a sequence between the targets.

5 Validation and verification

5.1 General considerations

The first conventional end-point mpPCR methods for GMO detection were developed about 20 years ago (Meyer, 1999; Matsuoka *et al*., 2001) and the approach has been recommended afterwards in other studies to simplify the multi-target analyses and to reduce corresponding efforts *(*Anklam *et al. 2002;* Holst-Jensen *et al. 2003;* Marmiroli *et al.* 2008). Nowadays, many mpPCR protocols are available, including published mpPCR methods for GMO screening. The qualitative mpPCR methods for GMO screening detecting P-35S, T-nos, ctp2-cp4-epsps, bar and pat sequences in duplex, triplex or pentaplex PCR assays are well-known examples (Waiblinger *et al*. 2008; Huber *et al.* 2013).

These studies also address specific requirements on developing and validating mpPCR methods. However, even already validated methods may pose difficulties when implementing them in another laboratory and using them for routine analysis. Problems could be caused by different laboratory equipment or modifications in the reaction setup (for example, the use of a different master mix, fluorophore or quencher due to the different instrument specifications) (Verginelli *et al.* 2020).

A prerequisite for the successful implementation of mpPCR methods in routine laboratory applications is a satisfactory optimisation and in-house validation. In the case of published mpPCR methods, data on the in-house validation or verification are often provided, sometimes even about interlaboratory validation studies. Thereby, the performance characteristics for specificity, dynamic range, R^2 coefficient, PCR efficiency, trueness, measurement uncertainty, limit of detection (LOD) and limit of quantification (LOQ) are available. Importantly, it is required for mpPCR to have data for the asymmetric LOD (LOD_{asym}).

Other starting points to establish a mpPCR method could be (i) the combination of existing singleplex PCR methods (Köppel and Zimmerli 2010) or (ii) the exchange or addition of a module in an existing mpPCR assay (Köppel *et al.* 2012). Specific considerations for in-house validation and verification in the case of removal, replacement and addition of one (or more) PCR module(s) in a mpPCR method are provided in section 6.

The parameters to be checked are different for qualitative and quantitative mpPCR methods (Figure 2). It is generally recommended to consider the 'Minimum Performance Requirements' (MPR) (ENGL, 2015) and the 'ENGL Verification' documents for guidance (Hougs *et al.* 2017). As usual, an in-house validation of a new mpPCR method has to be more extensive than a verification of an already validated method.

The performance of qualitative mpPCR methods with regard to **applicability**, **practicability**, **limit of detection (LOD), specificity, cross-talk** and **robustness** should be assessed, preferably according to established guidance documents (ENGL, 2015; BVL, 2016)**.** In addition, for quantitative methods, the **limit of quantification (LOQ)**, **dynamic range** and **accuracy** need to be evaluated. Some of these parameters should be tested in **asymmetric reaction conditions** (e.g. LOD and LOQ) in order to assess the absence of competition due to the concurrent amplification of the targets.

Single PCR modules complying with the performance requirements may not always perform satisfactorily, if included in a mpPCR method, particularly under asymmetric target DNA conditions. Therefore, it is recommended to use single PCR modules with optimised performance or to consider developing new modules for the underperforming targets. In general, an in-house validation of a new mpPCR method shall be performed.

The **applicability** of the multiplex method should be assessed (ENGL, 2015), i.e., the matrices (feed, food, raw/processed material) and DNA concentration ranges at which the method can be applied (e.g., LOD_{asym} and compatible amount and combinations of the targets). Warnings on interferences from other analytes and on the inapplicability to certain matrices and conditions should be reported.

Information regarding the **practicability** of the mpPCR method should be provided (ENGL, 2015). A highly complex multiplex method may require investment in a new expensive instrument and may reduce the ease of operations, feasibility and efficiency of implementation in a laboratory.

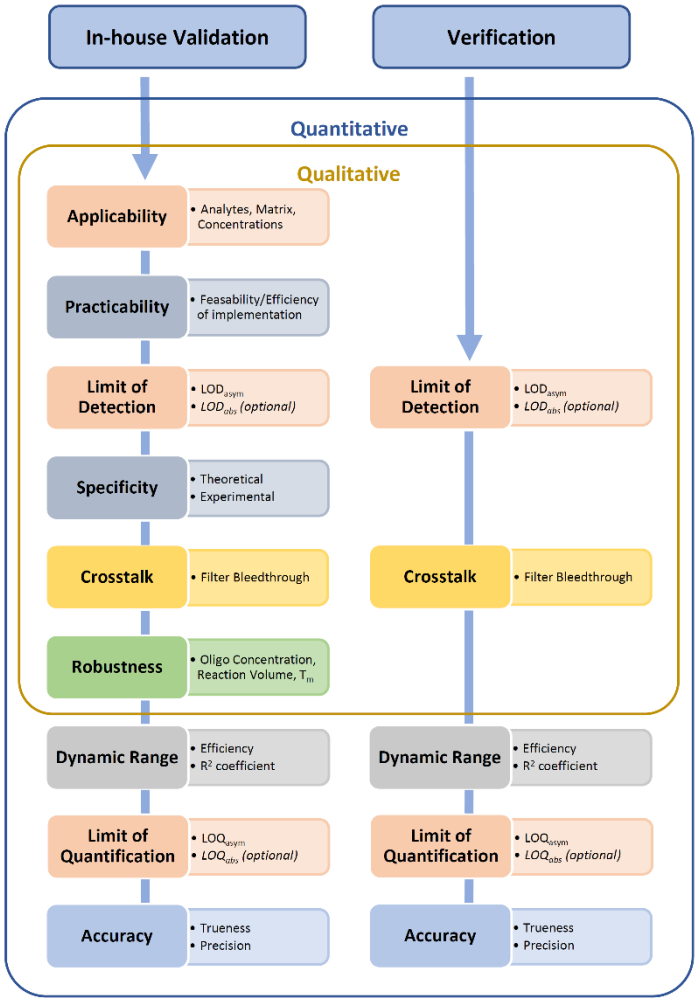

Figure 2 Illustration of the different performance parameters assessed during in-house validation or verification of qualitative and quantitative mpPCR methods.

5.2 Procedures for verification

The easiest starting point for a routine laboratory is to verify an existing and interlaboratory validated mpPCR method. Therefore, the requirements for method verification will be described below first.

If single PCR parameters are modified, for example the brand of a ready-to-use master mix or Taq polymerase, the PCR volume, the primer and probe concentrations or the PCR cycling parameters, selected performance parameters should be experimentally assessed (e.g. specificity and robustness). It is recommended to follow published guidelines (Woll *et al.* 2013; Hougs *et al.* 2017; ENGL Guidelines on the Update of GMO EURL GMFF Validated Methods, *in prepration*).

Note: If one or more PCR module(s) are removed from a multiplex method, a new method verification is not required.

5.2.1 Verification of qualitative multiplex PCR methods

The procedures and acceptance criteria of the relevant performance parameters to be assessed in a method verification are described in the following sub-chapters.

5.2.1.1 Limit of detection under asymmetric conditions (LOD_{asym})

PCR modules combined in a single reaction may be affected by competitive effects. Therefore, the capacity to detect small amounts of each target should be assessed in the presence of high amount(s) of the other target(s). For such situations, the LOD is defined as asymmetric LOD (LOD_{asym}).

Definition:

The LOD_{asym} is the lowest amount of an analyte in a sample that can be reliably detected, but not necessarily quantified, in the presence of high amount(s) of other target(s) in the mpPCR assay. The LOD_{asym} is expressed in absolute copies per reaction and is given for the corresponding amount of the other target(s) used in the test (e.g. 10 copies/reaction of target A in the presence of 1,000 copies/reaction of target B).

Procedure:

The LOD_{asym} should already have been investigated in the context of method validation (see section 5.3.1.1 for details in single laboratory validation). For verification of mpPCR targeting GM elements, constructs or events, each module should be tested for LOD_{asym} in the presence of the other target(s), according to the validation data. The magnitude of the highest amount of the other target(s) depends on the scope of the mpPCR (e.g. detection of GM or taxon-specific targets). Furthermore, the maximal amount of the other target(s) depends on the characteristics of the sample materials (i.e. genome size, zygosity, availability of material with high GMO content etc.) and on the maximal amount of total DNA that is compatible with the PCR method.

The laboratory should assess the LOD_{asym} for each target under the same asymmetric target conditions as reported in the original validation report. At least ten (10) PCR replicates should be tested and positive amplification signals for all replicates and for all targets should be observed. If this would not be the case, the laboratory should assess the LOD_{asym} according to section 5.3.1.1.

Example: the validation of a triplex mpPCR method reporting an LOD_{asym} of 10 copies/reaction of each target in the presence of 2,500 copies/reaction of all other targets. For each combination, ten (10) PCR replicates are tested at the LOD_{asym} condition (Table 3). All examined PCR replicates should provide positive PCR results.

Table 3 Example of tests for the determination of LOD_{asym} for all targets in a triplex-PCR (replicates at each level n=10).

Multiplex PCR Condition	Target sequence A (copies/reaction)	Target sequence B (copies/reaction)	Target sequence C (copies/reaction)
Test LOD_{asym} target A	10	2,500	2,500
Test LOD_{asym} target B	2,500	10	2,500
Test LOD_{asym} target C	2,500	2,500	10

Note 1: If one or more PCR replicates are negative, the concentration of the DNA sample used for the PCR module under verification should be checked (e.g. by digital PCR; or by real-time PCR in a simplified test for the PCR module under verification in absence of the other target(s), e.g. with 6 replicates of 6 dilution levels). If the DNA

concentration for the PCR module under verification is confirmed, the concentration of the more abundant targets can be reduced until all replicates give a positive signal.

Note 2: For the verification of mpPCR methods including a PCR module generally expected to be only applied at high copy number (e.g. taxon-specific module), the above described procedure is not applicable to that module. In such cases, verification of the LOD_{abs} of the specific module should be performed.

Note 3: The estimation of the LOD_{asym} is not carried out for mpPCR methods where the target sequences are detected simultaneously with an identical reporter dye. Alternatively, the LOD_{abs} should be verified for each target.

Note 4: The amount of other target(s) under asymmetric conditions depends on the scope of the multiplex method.

Acceptance criterion:

The LOD_{asym} corresponds to the lowest amount of target where all examined PCR replicates provide positive PCR results. The LOD_{asym} for each individual PCR module of a qualitative mpPCR method should be in line with the published validation data.

5.2.1.2 Cross-talk

Definition:

The fluorescence signals generated during the amplification of different targets may bleed-through between adjacent detection channels and superimpose each other. This effect is also known as cross-talk and may fake target sequence amplifications, resulting in false positives.

Note: Cross-talk is highly dependent on the dyes and the instrument settings chosen for the method and should be assessed during verification of qualitative and quantitative mpPCR methods. In general, it can be avoided by appropriate dye calibration settings of the instrument software and the selection of reporter dyes having distinct emission spectra far apart from each other (see section 3.2.2).

Procedure*:*

The absence of cross-talk may be examined with test samples containing no target DNA for the PCR module and the respective channel under investigation, but high target copy numbers for the other PCR module(s) and respective channel(s), e.g. 20,000 copies per channel (Table 4). The quantity of target sequences should at least correspond to the number of copies used in the determination of LOD_{asym}. Three replicates per testing condition are recommended.

Table 4 Experimental design of the cross-talk test for a multicolour tetraplex-PCR (replicates at each condition n=3)

Condition	**Target sequence A (copies/ reaction)**	**Target sequence B (copies/ reaction)**	**Target sequence C (copies/ reaction)**	**Target sequence D (copies/ reaction)**
Cross-talk A	0	20,000	20,000	20,000
Cross-talk B	20,000	0	20,000	20,000
Cross-talk C	20,000	20,000	0	20,000
Cross-talk D	20,000	20,000	20,000	0

Note 1: The cross-talk test assesses the leak of fluorescence emitted by the dyes (filter bleed-through) in the channel dedicated to a PCR module for which no target sequence is present. A fluorescence signal detected in the channel under investigation may also be caused by molecular "cross-hybridisation" of the probe at a target sequence of another PCR module. Each real-time PCR instrument has to be specifically checked for signal cross-talk of the channels. Internal quenchers are helpful to reduce the general fluorescence background and to improve

fluorescence signal separation. Consider also the possibility of contamination in the reference material used for the cross-talk test; this could be tested in a singleplex format.

Note 2: Molecular cross-talk (cross-hybridisation) between probes and unintended targets should already have been investigated in the context of method validation.

Note 3: The test for cross-talk is not required for mpPCR methods where the target sequences are detected simultaneously with an identical reporter dye in one channel.

Acceptance criterion:

No cross-talk should be detected. In certain circumstances cross-talk may be acceptable for the application, if the method is still fit for purpose. The possible cross-talk signal should be below the fluorescent threshold of the detection channel. The threshold should be below the positive control signal and above the highest cross-talk experienced. For example, a maximum cross-talk fluorescence of 10 % of the positive control may be acceptable.

Note: If the performance criterion is not met, an improvement may be reached by reducing the concentration of the 'cross-talking' probe or by changing the probe label. When a fluorescence threshold is set, the possible cross-talk signal shall not exceed this threshold and thus not cause a measurable Cq value. The fluorescence threshold should be set as under routine testing conditions for the respective PCR method.

5.2.2 Verification of quantitative multiplex PCR methods

The simultaneous amplification of different PCR targets makes the multiplex reaction a useful approach for GMO quantification.

In principle, quantification by mpPCR can be done by amplifying taxon-specific targets in combination with multiple GM-specific targets (event-, construct- or element specific targets). However, the most common application relies on duplex quantitative methods targeting one taxon-specific and one GM-specific target. An advantage of mpPCR quantitative methods is that each target is tested in the same reaction, avoiding differences due to different conditions between different tubes.

The present document is focused on this most common application of a quantitative mpPCR.

Validation and verification procedures should be performed in line with the expected application conditions. Therefore, the parameters should be assessed under asymmetric conditions, meaning that the GM-specific target(s) should be tested in the presence of an excess of the taxon-specific target(s).

This section addresses duplex quantitative PCR methods in which one of the targets is the taxon-specific gene and the other is the GM-specific target, but it is not limited to this format. Considering a duplex PCR method including the taxon-specific target, the possibility to create asymmetric conditions is limited by the nature of the material itself, in which the taxon-specific gene is present at a level of 100%.

In general, the procedures described in the ENGL Method Verification document (Hougs *et al.* 2017) and the acceptance criteria given therein apply also for quantitative mpPCR methods.

In addition to these general procedures for method verification, the performance of quantitative mpPCR methods should be verified with respect to the LOQ_{asym} and cross-talk.

5.2.2.1 Limit of quantification under asymmetric conditions (LOQ_{asym})

PCR modules combined in a single reaction may be affected by competitive effects. Therefore, the capacity to quantify small amounts of each target should be assessed in the presence of high amount(s) of the other target(s). For such situations, the limit of quantification (LOQ) is called asymmetric LOQ (LOQ_{asym}).

Definition:

The asymmetric limit of quantification (LOQ_{asym}) is the lowest amount of analyte in a sample that can be reliably quantified with an acceptable level of trueness and precision in the concomitant presence of high amount(s) of the other target(s) in the mpPCR assay.

Procedure:

The LOQ_{asym} should have already been investigated in the context of method validation (see section 5.3.2.1). For verification of mpPCR methods for GM element-, construct- or event-specific targets, each module should be tested for LOQ_{asym} in the presence of the other target(s), according to the validation data. The magnitude of the highest amount of the other target(s) depends on the scope of the mpPCR method and on the characteristics of the sample materials (i.e. genome size, zygosity, availability of material with high GMO concentration etc.).

The laboratory should assess the LOQ_{asym} for each target under the same asymmetric target conditions as reported in the original validation report. Different asymmetric conditions could also be tested.

For a duplex quantitative real-time PCR method (i.e. GM assay and taxon-specific assay), relative GM quantification of samples at a low GM content is performed by definition in asymmetric conditions by targeting low amounts of the GM-target in a high background of the taxon-specific target (e.g. testing 0.1% material).

Alternatively, the LOQ_{asym} can be estimated in absolute copy numbers per reaction by testing an amount of the GM target of less than or equal to 50 copies per reaction in the presence of an amount equal to or higher than 56,000 copies per reaction of the taxon-specific target (ENGL, 2015). The amount of the taxon-specific target could be lower in case of species having a large genome size.

In both approaches, each sample or dilution level should be analysed in at least 10 PCR replicates around the expected LOQ_{asym}. The performance requirements for precision (RSD_r) and trueness (bias) have to be fulfilled according to the acceptance criteria.

Example: A sample at the expected LOQ_{asym} is prepared according to the validation report of the method and tested in 10 PCR replicates.

Table 5 Examples for determination of the LOQasym in copies/reaction of the GM target in a duplex-PCR (replicates at each level n=10).

GM target sequence (copies/reaction)	**Taxon-specific target sequence (copies/reaction)**
50	56,000

Acceptance criteria:

The LOQ_{asym} should be below or equal to the lowest amount included in the dynamic range and should correspond to the last dilution level showing a level of precision of $RSD_r \leq 25\%$ and trueness within a bias of ± 25 % of the reference value.

The LOQ_{asym} for each individual PCR module of a quantitative mpPCR method should be in line with the validation report and with the MPR guidelines.

For quantitative duplex PCR methods (i.e. GM assay and taxon-specific assay), the LOQ_{asym} should be verified at a level ≤ 0.10 % (m/m).

In terms of absolute copy number per reaction, the LOQ_{asym} should be ≤ 50 copies of the GM target in the presence of at least 56,000 copies of the taxon-specific target, in line with the requirements for dynamic range of the MPR.

However, for quantitative taxon-specific methods used in combination with a GM-specific method, the LOQ_{abs} should be assessed.

5.2.2.2 Cross-talk

Cross-talk should be assessed as described in section 5.2.1.2.

5.2.2.3 Dynamic Range, Amplification efficiency and R^2 coefficient

The method should be assessed to provide reliable results within the range of content values corresponding to the expected use. Within the dynamic range, the standard curves should meet the acceptance criteria for the amplification efficiency and the coefficient of determination (R^2).

Procedure:

Dynamic range, amplification efficiency and R^2 coefficient can be verified simultaneously from standard curves generated under asymmetric conditions when testing other parameters, such as trueness and precision. Five calibration points are analysed in at least two PCR replicates. The average values of at least two standard curves should be considered.

When verifying a quantitative duplex PCR method targeting a GM-target and the taxon-specific target, a dilution series is prepared from the DNA sample starting from the highest relative GM content. The range of copies to be covered is specified in the MPR and corresponds to at least 50 to 2,520 copies/reaction for the GM PCR module and 50 to 56,000 copies/reaction for the taxon-specific PCR module. For practical reasons an asymmetric ratio of 1/10 for the calibration material (e.g. Certified Reference Material (CRM) GM level of 10 %) is recommended. However, different asymmetric conditions could be used.

Table 6 Example of the determination of dynamic range, R^2 coefficient and amplification efficiency of a quantitative duplex PCR method targeting a GM and the taxon-specific targets (replicates at each level n≥2).

Target sequence A (GM) (copies/reaction)	**Target sequence B (e.g. taxon) (copies/reaction)**
6,000	60,000
1,000	10,000
200	2,000
50	500
20	200
5 (Not Analysed)	50

Acceptance criteria:

The average value of the slope of the standard curve shall be in the range of -3.6 ≤ slope ≤ -3.1, corresponding to an amplification efficiency of 90 - 110 %.

The average value of R^2 of the standard curves shall be ≥ 0.98.

5.2.2.4 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.

Procedure:

The trueness should be determined at a content close to the level set in legislation (e.g. threshold 0.9 % m/m), or according to the intended use of the method, and, if appropriate, additionally at a level close to the LOQ. The trueness can be assessed by using a CRM. Two mass fractions (e.g. 0.1 % and 1 % m/m) and, if possible, a third mass fraction at the upper end of the dynamic range (e.g. 5 % m/m) should be investigated. Alternatively, a reference sample may be prepared, preferably from a higher concentrated CRM (see Annex 3 in Hougs *et al.*, 2017).

The analytical procedure used, including reaction volume, PCR instrument etc., should be the same as during routine testing of samples. Results from at least 15 PCR replicates should be evaluated. The GM content is calculated from each single PCR replicate (see Annex 4 in Hougs *et al.*, 2017).

Example 1: test 2 DNA extraction replicates per GM level, 2 PCR replicates per extraction/plate, 4 plates resulting in 16 GM-content estimations per GM level.

Example 2: test 2 DNA extraction replicates per GM level, 4 PCR replicates per extraction/plate, 2 plates resulting in 16 GM-content estimations per GM level.

If CRMs for estimating the trueness are not available, a sufficiently characterized proficiency test material can be employed, if its stability is ensured.

Acceptance criteria:

For all the GM levels tested the trueness should be within ± 25 % of the accepted reference value.

5.2.2.5 Precision - Relative Repeatability Standard Deviation (RSD_r)

Definition:

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

Procedure*:*

Precision expressed in terms of relative repeatability standard deviation (RSD_r) can be determined in a similar way as already described for trueness (5.2.2.4). RSD_r is calculated from the results obtained on PCR replicates analysed under repeatability conditions during the estimation of trueness. Repeatability data should be available for all the GM levels tested.

The analytical procedure used should be the same as during routine testing of samples. At least 16 single test results should be evaluated.

Acceptance criterion:

The RSD_r should be ≤ 25 % for all the GM levels tested.

5.3 Procedures for in-house validation

A new mpPCR method has to be validated in-house before its implementation for routine use in the laboratory (and its potential further validation by an interlaboratory study). This is the case, if combining:

- several validated singleplex PCR methods,
- validated singleplex PCR module(s) with newly developed PCR module(s),
- newly developed PCR modules in a mpPCR method.

The procedures for the validation experiments are described for qualitative and quantitative methods in the following sections.

Applicability and practicability are not addressed here, but should be assessed and described in the validation report of the method (see section 5.1).

Note: If one or more PCR module(s) are removed from a validated mpPCR method, a new in-house validation is not required.

5.3.1 In-house validation of qualitative methods

In the following sub-chapters, the procedures and acceptance criteria of the different relevant performance parameters for assessment of in-house validation are described.

5.3.1.1 Limit of detection under asymmetric conditions (LOD_{asym})

For in-house validation of the LOD_{asym} it is required to follow the general considerations for verification as described in section 5.2.1.1. However, a larger number of replicates and content levels should be tested.

Note: The LOD_{abs} under multiplex conditions in the absence of the other target(s) can optionally be assessed separately for each PCR module.

Definition:
See section 5.2.1.1

Procedure:

For the validation of a mpPCR method for the detection of GM element-, construct- or event-specific targets, each module should be tested for LOD_{asym} in the presence of high amounts of the other target(s). The magnitude of the amount of the other background target(s) depends on the scope of the mpPCR, the characteristics of the reference materials (i.e. target copy number, zygosity, availability of material with high GMO level etc.) and on the maximum amount of total DNA that is compatible with the PCR method. At least 2,500 copies/reaction of each of the other targets are recommended, however, higher amounts should be considered depending on the scope of the method (e.g. GM and taxon targets in multiplex format).

The determination of the LOD_{asym} of each module of a multiplex-PCR may be established by means of serial dilutions made from mixtures of the target DNA (genomic DNA, plasmid DNA or amplicon DNA) in excess of the other target(s).

In order to reach the required level of confidence (i.e. ≤ 5% false negative results), a suitable number of replicates has to be tested. According to the MPR document (ENGL, 2015), a suitable number of replicates per amount may be 60, with the LOD_{asym} set at the lowest amount yielding at least 59 positive results.

As this may not be feasible, a pragmatic approach based on a lower number of replicates could be followed which allows an approximate estimation. The LOD_{asym} can be assessed

on dilution levels of each target in excess of the other target(s) and tested in at least 12 replicates per level (CEN, 2019). The LOD_{asym} is the lowest amount at which all examined PCR replicates show positive PCR results for all targets.

Example: For a triplex-PCR, test several dilution levels (e.g. 50, 25, 10, 5, 1, 0.1 copies/reaction) and 12 replicates per level in the presence of 2,500 copies per reaction of the other targets (Table 7).

Table 7 Example for the determination of LOD_{asym} of one target in a triplex-PCR (replicates at each level n=12)

Target sequence A (copies/reaction)	Target sequence B (copies/reaction)	Target sequence C (copies/reaction)
50	2,500	2,500
25	2,500	2,500
10	2,500	2,500
5	2,500	2,500
1	2,500	2,500
0.1	2,500	2,500

See *Annex B: Estimation of the asymmetric limit of detection* for further examples in dependence on reference material availability.

Note 1: For the validation of mpPCR methods including a PCR module usually present at high copy numbers (e.g. taxon-specific module), the above described procedure is not applicable for that module. In such case, it is recommended to perform LOD_{abs} validation without the presence of the other target(s).

Note 2: If one or more PCR replicates are unexpectedly negative, the concentration of the DNA sample used for the PCR module under validation should be checked (e.g. by digital PCR; or by real-time PCR in a simplified test for the PCR module under multiplex conditions in the absence of the other target(s), e.g. with 6 replicates of 6 dilution levels). If the DNA concentration for the PCR module under validation is confirmed, the concentration of the more abundant target(s) can be reduced until all replicates give a positive signal.

Note 3: The estimation of the LOD_{asym} is not carried out for mpPCR methods where the target sequences are detected simultaneously with an identical reporter dye.

Note 4: The absolute LOD under multiplex conditions in absence of the other target(s) can optionally be assessed separately for each PCR module.

Acceptance criterion:

The LOD_{asym} for each individual PCR module of a qualitative mpPCR method should be < 25 copies/reaction in the presence of a high copy number of other target(s) with a level of confidence of 95%, ensuring ≤ 5% false negative results. The amount of other target(s) under asymmetric conditions depends on the scope of the multiplex method. A minimum amount of other targets could be limited by the availability of reference materials and the total amount of DNA compatible with the mpPCR method. It is recommended to use at least 2,500 copies of each of the other background targets when testing an LOD under asymmetric conditions.

5.3.1.2 Specificity

The specificity of a new mpPCR method to respond exclusively to the individual target sequences should be evaluated by theoretical (bioinformatics) *in silico* tests and in experimental PCR tests during validation. The assessments aim at confirming:

- that each target is detected,

- the compatibility of primers and probes (e.g. melting temperature, primer dimer formation, hairpin formation),
- the absence of unexpected/unwanted non-target amplifications due to the presence of several primers and probes in one reaction.

In silico Specificity

In order to predict potential false positive results in multiplexing, all possible combinations of oligonucleotide sequences (primer, probe) should be evaluated in a computer-aided (*in silico*) test for compatibility between primers and similarities to sequences of other genomes relevant for the field of application of the method.

Procedures:

Primers and probes compatibility

To verify the compatibility of primers and probes, it is necessary to analyse different features such as melting temperature (T_m) of the oligonucleotides, secondary structures and cross-dimer formation between all the primers included in the multiplex test. To achieve this, several programs can be applied. Most programs used to test the compatibility for a single target PCR are suitable. The test has to be realised for all the combinations of primers (see Annex C: *In silico* specificity assessment).

Checking the specificity by *in silico* PCR

This step will search the possible similarities against a database of sequences to confirm the amplification of the expected sequences and highlight possible unwanted/unexpected amplifications. The databases should comprise a large collection of conventional DNA sequences (e.g. NCBI[2], ENA[3]) and specific GMO sequences[4] (Petrillo *et al.*, 2015; Ye *et al*. 2012). Databases have to contain plant genomes, GMO sequences, but also the donor organisms of the structural elements.

The main programs used to perform this *in silico* PCR are briefly compared in Table 22.

Performance criteria:

The *in silico* analysis should not identify any unexpected similarities to other target sequences. The differences between the sequences (of primer vs. unintended targets) should be at least two mismatches and two gaps per primer. Different software tools are based on different algorithms for assessing the possible formation of artefacts. For this reason, any "acceptance value" is programme-specific and no general threshold or score can be given here.

Experimental Specificity

In order to check whether additional amplicons or artefacts are formed due to the combination of several primers and probes in the mpPCR, the method should be tested with material sets reacting positive as well as negative for the targets. Additionally, the PCR products should be examined experimentally by demonstrating the absence of amplification products.

In case of similarity to other targets identified by *in silico* analysis, experimental confirmation is required to assess possible unspecific amplification products.

Procedure:

The tests should be conducted with approximately 2,500 copies of non-target DNA and with at least 100 copies of target DNA. A minimum of 2 replicates per test is recommended.

[2] National Center for Biotechnology Information https://www.ncbi.nlm.nih.gov/
[3] ENA - European Nucleotide Archive https://www.ebi.ac.uk/ena/browser/search
[4] Collection of nucleic acid sequences related to genetically modified organisms https://data.europa.eu/euodp/data/dataset/fe7a9cc6-6100-4365-89d7-6e09d1f570cf

The setup should consist of testing non-target materials (matrices that are relevant for the laboratory, e.g. maize, soy, canola, feed, mixed feed, food) and target materials (e.g. samples with different matrices and GMO content) (ENGL, 2015).

The specificity may further be checked by confirming the identity of the amplified sequence by amplicon sequencing. Gel electrophoresis or capillary electrophoresis can be used to check the generation of unexpected additional amplicons.

Note: cross-talk may also provide unspecific fluorescence signals and should be checked, if required (see section 5.2.1.2)

Note: matrix reference materials are only certified for the presence or absence of a specific GM event and not for the absence or presence of other GM events that could be present as trace constituents. Information on contaminants in reference materials are shared by the European GMO Initiative for a Unified Database System[5].

Acceptance criteria:

All PCR results have to correspond to the theoretical expectations.

If any cross-reactivity (including the formation of unspecific amplicons or artefacts) are deemed acceptable for the application of the method, they have to be indicated and taken into account for the scope of the method.

5.3.1.3 Cross-talk

For the in-house validation of the cross-talk it is required to follow the general considerations and procedures as described in section 5.2.1.2.

5.3.1.4 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.

Procedure:

Robustness should be assessed in order to mimic the operational implementation of the method. The method should be able to provide accurate results in cases when small deviations to the experimental protocols are introduced.

As opposed to singleplex PCR methods, several factors are not amendable because of technical reasons. For instance, two different real-time PCR devices and reagent kits applicable to mpPCR might not be available in the laboratory. In addition, the number of conditions related to primer and probe concentrations increases with the number of individual PCR modules.

Relevant factors and appropriate PCR assay deviations should be tested using two different conditions. Various parameters of interest are varied over a specified range (i.e. decreased and increased, respectively). For a tetraplex PCR method, factors that should be tested are indicated in Table 8. Primer and probe concentrations of every module should be regarded as independent factors that are added individually to the PCR mix.

For testing the robustness of the mpPCR method a multi-factorial study design approach testing several changes at once is recommended (ENGL, 2015; Uhlig *et al.*, 2015; BVL, 2016). Examples of multifactorial studies for mpPCR methods are provided in Annex D: Examples of practical settings for robustness testing.

[5] European GMO Initiative for a Unified Database System https://euginius.eu/euginius/pages/home.jsf

Note: further multi-factorial designs for additional factors or higher degrees of multiplexing can be adapted from the Tables of Taguchi Designs (Orthogonal Arrays, see Taguchi and Konishi, 1987)[6].

Table 8 Examples of conditions tested during robustness assessment of a tetraplex PCR method

Factor	**Condition 1**	**Condition 2**
Annealing temperature	- 1 °C	+ 1 °C
Reaction volume	- 1 µL	+ 1 µL
Primer concentration module 1	- 10 %	+ 10 %
Probe concentration module 1	- 10 %	+ 10 %
Primer concentration module 2	- 10 %	+ 10 %
Probe concentration module 2	- 10 %	+ 10 %
Primer concentration module 3	- 10 %	+ 10 %
Probe concentration module 3	- 10 %	+ 10 %
Primer concentration module 4	- 10 %	+ 10 %
Probe concentration module 4	- 10 %	+ 10 %
Master mix concentration	- 10 %	+ 10 %

Acceptance criterion:

The method should always give the expected results in terms of presence of the targets under the tested conditions.

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

5.3.2 In-house validation of quantitative multiplex PCR methods

This chapter is primarily focused on duplex quantitative PCR methods. More complex mpPCR methods may require an adaptation from the described procedures.

5.3.2.1 Limit of quantification under asymmetric conditions (LOQ_{asym})

For an in-house validation of the LOQ_{asym} it is required to follow the general considerations described in section 5.2.2.1. However, a larger number of replicates and concentration levels should be tested for the in-house validation.

Note: the absolute LOQ (LOQ_{abs}) under multiplex conditions in absence of the other target(s) may also be assessed separately for each PCR module.

Definition:
See section 5.2.2.1

Procedure:

For the validation of a mpPCR method for the quantification of GM element-, construct- or event-specific targets, each module should be tested for LOQ_{asym} in the presence of high amounts of the other target(s). The magnitude of the amount of the other background target(s) depends on the scope of the mpPCR, the characteristics of the reference materials

[6] Orthogonal Arrays (Taguchi Designs) https://www.york.ac.uk/depts/maths/tables/orthogonal.htm

(i.e. genome size, zygosity, availability of material with high GMO level etc.) and on the maximum amount of total DNA that is compatible with the PCR method.

For a quantitative duplex mpPCR method (i.e. GM assay and taxon-specific assay), a quantification of samples with low GM content is performed by default under asymmetric conditions by targeting the low amount of the GM-target in a high background of the taxon-specific target (e.g. testing 0.1% (m/m) material).

In order to reach the required level of $RSD_r \leq 25$ %, a suitable number of replicates should be tested. The LOQ_{asym} can be estimated on a dilution series of one target in a high background amount of the other target(s). Each dilution level should be analysed in at least 15 PCR replicates (ISO 5725-2; ENGL, 2015).

Example:

A dilution series is made from DNA under asymmetric conditions. The dilution levels have a low content of GM target DNA (e.g. 50, 40, 20, 10 copies per reaction) and at least 56,000 copies per reaction of the taxon-specific target.

Table 9 Example for the determination of LOQ_{asym} of one target in a quantitative duplex-PCR (replicates at each level n=15)

GM target sequence (copies/reaction)	Taxon-specific target sequence (copies/reaction)
50	56,000
40	56,000
20	56,000
10	56,000

Acceptance criteria:

The LOQ_{asym} should be below or equal to the lowest amount included in the dynamic range and should correspond to the last dilution level showing a level of precision of $RSD_r \leq 25$ % and trueness within a bias of ± 25 % of the reference value.

The LOQ_{asym} for each individual PCR module of a quantitative mpPCR method should be in line with the MPR document.

For combined modules (quantitative duplex) the LOQ_{asym} should be ≤ 0.09 % (m/m).

For individual modules, the LOQ_{asym} should be ≤ 50 copies in the presence of high amounts of other target(s) (with a level of confidence of 95 %). The amount of other target(s) depends on the scope of the multiplex method. For quantitative duplex mpPCR (i.e. GM assay and taxon-specific assay), at least 50 copies of the GM-target should be quantifiable in the presence of at least 56,000 copies per reaction of the target of the taxon-module. In other cases, it is recommended to determine the LOQ_{asym} with at least 2,500 copies per reaction of the other target.

5.3.2.2 Specificity

Specificity should be assessed as described in section 5.3.1.2.

5.3.2.3 Cross-talk

Cross-talk should be assessed as described in section 5.2.1.2.

5.3.2.4 Robustness

Robustness should generally be tested as described in section 5.3.1.4.

Factors that should be tested for a duplex PCR method are given in Table 10.

Table 10 Examples of conditions tested during robustness assessment of a duplex PCR method

Factor	Condition 1	Condition 2
Annealing temperature	- 1 °C	+ 1 °C
Reaction volume	- 1 µL	+ 1 µL
Primer concentration module 1	- 10 %	+ 10 %
Probe concentration module 1	- 10 %	+ 10 %
Primer concentration module 2	- 10 %	+ 10 %
Probe concentration module 2	- 10 %	+ 10 %
Master mix concentration	- 10 %	+ 10 %

Procedure:

In order to evaluate the robustness of a real-time PCR method, different experimental conditions should be slightly modified and their impact on the results has to be examined.

Method robustness should be evaluated under asymmetric conditions at the LOQ_{asym}. For each combination of modifications, the LOQ_{asym} should be tested in at least 3 PCR replicates. In order to validate the other PCR module(s) as well, the test should be repeated with modified amounts for the modules. For applying the factorial design approach an example including PCR plates setup is provided in Annex D: Examples of practical settings for robustness testing

In order to calculate copy numbers or mass fractions from Cq values, standard curves have to be measured in a quantitative duplex PCR robustness test. For the sake of simplicity, only one standard curve under optimal (unchanged) conditions, except the annealing temperature, may be used per PCR plate (see Figure 3 in Annex D).

Acceptance criteria:

The quantification of the sample at the LOQ_{asym} should show a bias of not more than ± 30 % and an RSD_r ≤ 30 % when any combination of modifications is tested.

Note: if unacceptable 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.

5.3.2.5 Dynamic Range, Amplification efficiency and R^2 coefficient

The method should be assessed to provide reliable results within the range of content values corresponding to the expected use. Within the dynamic range, the standard curves should meet the acceptance criteria for the amplification efficiency and the coefficient of determination (R^2).

Definitions:

See section 5.2.2.3

Procedure:

Dynamic range, R^2 coefficient, and amplification efficiency are verified simultaneously from standard curves when testing other parameters, such as trueness and precision. The dynamic range is established on the basis of a standard curve tested on a minimum of four content levels evenly distributed and measured at least in duplicate. The amplification efficiency and R^2 should be assessed by at least 5 individual runs (ENGL, 2015).

These parameters should be assessed on standard curves generated under asymmetric conditions. When validating a quantitative duplex method targeting a GM-target and the

taxon-specific target, a dilution series with at least four content levels is prepared from the DNA sample at the highest relative GM content. The ranges of copies to be covered are those identified in the MPR and correspond to 50-2,520 copies/reaction for the GM PCR module and 50-56,000 copies/reaction for the taxon-specific PCR module. For practical reasons, an asymmetric ratio of 1/10 for the calibration material is recommended. However, different asymmetric conditions could be used.

Acceptance criteria:

Amplification efficiency: for each target the average value of the slope of the standard curve should be in the range of -3.6 ≤ slope ≤ -3.1, corresponding to an amplification efficiency of 90 - 110 %.

R^2 coefficient: the average value of R^2 shall be ≥ 0.98.

5.3.2.6 Trueness

Definition:

See section 5.2.2.4

Procedure:

Trueness and precision are verified simultaneously on sets of samples representing the dynamic range.

At least three content levels should be investigated: (1) a level close to that set by legislation; (2) a level close to the LOQ; (3) a level close to the upper part of the dynamic range. The trueness can be assessed by using a CRM or by spiking to obtain a sample with asymmetric amounts of the different targets.

Samples corresponding to three different content levels that span the whole dynamic range (e.g. 0.09 % m/m, 0.9 % m/m and 4.5 % m/m) should be analysed. For each level, at least 15 replicates should be tested under varying conditions (e.g. different days or operators).

Acceptance criterion:

The trueness should be within ± 25 % of the accepted reference value.

5.3.2.7 Precision: Relative Repeatability Standard Deviation (RSD_r)

Definition:
See section 5.2.2.5
Repeatability can be calculated from results obtained on PCR replicates measured under repeatability conditions during the estimation of trueness. Repeatability addresses every tested GM-content level.

Procedure:
Samples corresponding to three different mass fraction levels that span the whole dynamic range (e.g. 0.09 % m/m, 0.9 % m/m and 4.5 % m/m) should be analysed. For each level, at least 15 replicates should be tested under repeatability conditions.

Acceptance criterion:

The RSD_r should be ≤ 25 % over the dynamic range of the method.

6 Additional considerations

MpPCR methods are composed of several PCR modules each targeting a specific DNA sequence. Such modules could be removed, substituted, or added. In some cases, the amplification signals from all modules are measured in one channel and consequently the source of a possible positive signal has to be verified further. The preferred strategy depends on the purpose of the analysis. In this section, some guidance for these considerations is given.

6.1 Making major changes

A: Removal of PCR modules

It is expected that the removal of a single PCR module (or a number of PCR modules) from a mpPCR method should not negatively affect the performance of the remaining PCR modules. According to experience, the performance of a mpPCR method in which one PCR module is removed is not affected compared to the original, more complex, multiplex methods.

B: Addition of PCR modules

Increasing the complexity by the combination of two or more PCR modules may negatively affect the performance of the individual PCR modules in the resulting mpPCR method. A validation of all method performance criteria (for example, specificity, sensitivity and PCR efficiency) should be performed for each module under the multiplex conditions (as described in section 5.3).

C: Replacing PCR modules

The replacement of one PCR module from an mpPCR method with another one can lead to reduced performance of the resulting mpPCR method (as in situation B). Therefore, such a change should be considered as a new mpPCR method, meaning that all method performance criteria have to be validated again.

6.2 Use of single colour vs. multi-colour probes

Principle of single-colour mpPCR methods

In view of the speed of GMO developments and authorisations in the EU, the use of single-colour mpPCR methods may be relevant. In a single-colour mpPCR method, all probes in the reaction mix are labelled with the same fluorophore or with different fluorophores causing close-by emission wavelengths. A negative analytical result would mean that none of the targets is detected in the sample. However, it is not possible to determine the origin of a positive signal.

The use of this approach is particularly relevant for assessing the presence of different GMOs without aiming to identify each of them individually. Different strategies may be investigated for the development of such methods, related to different goals, such as EU regulatory status of the GMOs or their presence on the market. For instance:

- Targeting several GMOs which are not authorised in the EU. A positive signal is sufficient to conclude that the product is non-compliant with EU legislation: at least one unauthorized GMO is present among the targets. The specific event(s) that are present in the sample can then be determined by applying the event-specific methods in the singleplex format.
- Targeting several GMOs that are authorised in the EU, especially for quantification. This approach has been used in a single-colour multiplex droplet digital PCR allowing the quantification of 15 genetically modified lines of soybean (11 authorised and 4 with a pending authorisation at the date of publication) (Košir et al. 2017).

— Screening several GMOs which are very rarely detected. As the results are expected to be mostly negative, it is a way to reduce operational costs for the control of GMOs. Nevertheless, it may be necessary to run event-specific PCR methods to assess the presence of each of the targeted GMOs in case of a positive result.

Differences and similarities between single-colour and multi-colour multiplex real-time PCR

A single-colour mpPCR method is much faster and more cost-effective to validate and implement in laboratories than a multi-colour mpPCR. In fact, the experimental validation is to a large extent similar to the assessment of a singleplex PCR. Indeed, many challenging issues of multiplexing such as cross-talk and limit of detection under asymmetric conditions are not relevant. The limit of detection can be assessed by testing serial dilution of each target without the need of setting asymmetric conditions. The cross-talk does not need to be assessed because a single detection channel is used. Furthermore, performing a single-colour mpPCR method does not require equipment able to detect via multiple channels.

Similarly to multi-colour mpPCR, the concentration of PCR probes for single-colour mpPCR methods is higher than in singleplex PCR. Moreover, every probe is absorbing and emitting light at the same wavelengths, respectively, which could lead to a very low signal-to-noise ratio. It is therefore highly recommended to use non fluorescent quenchers or even double-quenched probes to reduce background fluorescence.

Potential primer/probe interactions may impair the amplification and may reduce PCR efficiency (see Annex C: In silico specificity assessment). These interactions are often too weak to prevent a proper amplification in qualitative assays, but may affect a quantification. For example, when different combinations of primers and probes from different modules are able to bind to non-target regions, more than one amplicon per cycle could be generated.

7 Troubleshooting

In this chapter the most common problems are summarised that may be encountered in establishing mpPCR methods and a list of possible solutions is provided. The list is not exhaustive and is intended for those laboratories who are establishing an already developed and validated mpPCR method. However, the situations described may arise also in the phase of development of a new method.

In case of an observed problem, it is advised to repeat the experiment for verifying that the problem is not accidental (e.g. missing a reagent).

Table 11 List of common problems and possible solutions

Appearance	Solution
No signal in one or more channels	Did you use a PCR master mix for multiplex applications? Was this PCR master mix recommended and/or tested? Operators should be aware that PCR master mixes should be compatible with multiplex applications. Was the cycling protocol (including the ramp rate) correct? Check that the PCR master mix fits to the program (e.g. activation time/temperature). Was the signal detection at the correct thermal cycle step correctly selected? Do the sequences and labels of the primers and probes correspond to the required ones? Are the dilutions of primers and probes correct? Master mixes designed for fast PCR should be cycled according to the instructions. Does your thermal cycler meet the fast cycling conditions? Did you add the correct template DNA? Was the template DNA-concentration correctly diluted and measured? Is the template DNA free of PCR inhibitors (e.g. ethanol residues) and not degraded? Were all components added in the correct concentration and volume? Check the reaction mix preparation.
Only one signal	Did you add all required probes with the correct fluorescence dyes? Did you activate all required channels for data measurement? Did you add all different template DNAs? Are the concentrations of the template DNA reasonable for the PCR set up? Are the target concentrations extremely asymmetric?
Poor signal strength	Were the DNA solution and the other components carefully mixed before pipetting? Are the probes old or were they exposed to light for a long period? Was the template DNA frozen and thawed several times? We recommend short-time storage of template DNA at 4 °C and long term at -20 °C. For long templates we additionally recommend glycerol addition (Rossmanith et al. 2011). Are the probes stored correctly? Verify that the storage conditions were in the dark and at an appropriate temperature. Are the concentrations of the probes and primers correct? If the amplification takes place late in the cycling phase, check for the template DNA concentration. Do the channels correspond with the spectral characteristics of the probes? If a thermal cycler requires a reference dye: check that no probes carry a label with the same excitation/emission wavelength (e.g. if ROX is used as reference dye, ROX or Texas Red are not suitable for labelling of probes). Is the thermal cycler correctly calibrated for the dyes used?
Cross-talk	Is your real-time PCR thermal cycler designed for multiplex applications? Do the channels correspond with the spectral characteristics of the probes?

Appearance	Solution
Asymmetric amplification underperforming	Is the asymmetry of the template DNA extreme? Try to reduce the concentration of the more abundant target(s) and check that the target tested at lower concentration is above the LOD. If high concentrations of primers are used, a competition among modules may take place in cases where conditions change (e.g. $MgCl_2$ concentration). Try to reduce primer concentrations for an optimal amplification. (Probably when 2 singleplex modules are combined, the concentration of the primers should be reduced to avoid competitions)

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List of abbreviations

CRM: Certified reference material

DNA: Deoxyribonucleic acid

dPCR: digital PCR

GMO: Genetically modified organism

LOD: Limit of detection

LOD_{asym}: Limit of detection under asymmetric conditions

LOQ: Limit of quantification

LOQ_{asym}: Limit of quantification under asymmetric conditions

mpPCR: multiplex real-time PCR

MPR: Definition of minimum performance requirements for analytical methods of GMO testing

PCR: Polymerase Chain Reaction

R^2: coefficient of determination

RSD_r: Relative repeatability standard deviation

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Figure 1 Schematic illustration of different approaches for mpPCR. The multiplex conventional end-point PCR method is based on the detection of amplicons of different size in horizontal or capillary electrophoresis (left). Multiplex real-time PCR or dPCR methods are based on the separate detection or quantification of the fluorescence from probes labelled with different fluorescent reporter dyes (right). 7

Figure 2 Illustration of the different performance parameters assessed during in-house validation or verification of qualitative and quantitative mpPCR methods. 15

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Annex A: DNA control stock solutions

DNA solutions are not stable at low concentrations (Ellison et al. 2006). Therefore, it is recommended to prepare stock solutions at high concentrations or dilute in background of for example non-target DNA (e.g. salmon sperm DNA). For multi-colour mpPCR, it is possible to mix several DNA controls in a single stock solution since the method will allow detection of each individual target.

An example for the preparation of a high concentrated genomic DNA stock solution for a triplex PCR is presented in Table 12.

Table 12 Example of the preparation of a positive control at 2,000 copies/uL for each of the target of a triplex mpPCR

Target	Initial target DNA concentration (copies/µL)	Final target DNA concentration (copies/µL)	Calculation of the Volume to add (µL)	v_{total}= 1000 µL (µL)
Target 1	30,000	2,000	2,000/30,000 * v_{total}	66.7
Target 2	20,000	2,000	2,000/20,000 * v_{total}	100
Target 3	43,000	2,000	2,000/43,000 * v_{total}	46.5
Diluent	-	-	$v_{total} - (v_1+v_2+v_3)$	786.8

If the volume of all individual solutions is higher than the total volume, the concentration of one or several individual DNA extracts is not high enough. In the example given in Table 13, the initial concentrations of targets 2 and 3 are too low. It is not possible to prepare a mixed solution at 10^4 copies/µL from these solutions. In such a case, it is necessary to prepare a mixture from more concentrated stock solutions or to reduce the final concentration (Table 13).

Table 13 Example of the preparation of a positive control for a triplex mpPCR

Target	Initial target DNA concentration (copies/µL)	Final target DNA concentration (copies/µL)	Calculation of the Volume to add (µL)	v_{total}= 1000 µL (µL)
Target 1	20,000	2,000	2,000/20,000 * v_{total}	100
Target 2	4,000	2,000	2,000/4,000 * v_{total}	500
Target 3	4,000	2,000	2,000/4,000 * v_{total}	500
Diluent	-	-	$v_{total} - (v_1+v_2+v_3)$	-100

Considering the preparation of a given volume (v_{tot}) of positive control for n-plex PCR. The positive control solution should contain *n* different targets with a given concentration ($c_{n,f}$). The volume v_n, taken from each individual stock solution, is proportional to the ratio between the stock solution concentration ($c_{n,0}$) and the final concentration, calculated as:

$$v_n = \frac{c_{n,f}}{c_{n,0}} \times v_{total}$$

The n-volume solutions will be mixed together with a volume v_w of diluent (e.g. water, buffer, TE, non-target DNA, etc.) obtained as difference between the sum of the volume from the positive samples (Σv_n) and the final volume v_{tot}:

$$v_w = v_{tot} - \sum v_n$$

v_{tot} and $c_{n,f}$ are set by the operator.

Annex B: Estimation of the asymmetric limit of detection

For in-house estimation of the LOD_{asym} each module should be tested in the presence of high amounts of the other target(s) as described in section 5.3.1.1. The magnitude of the amount of the other background target(s) is dependent on the characteristics of the reference materials (i.e. genome size, zygosity, availability of material with high GMO level etc.) and on the maximum amount of total DNA that is compatible with the PCR method.

This Annex is intended to show further examples for the determination of LOD_{asym} in triplex- and tetraplex-PCR methods in dependence on reference material characteristics and availability.

Table 14 Example 1. Determination of LOD_{asym} of one target in a triplex-PCR method (replicates at each level n=12). Here, all target sequences are from heterozygous GM maize with an available reference material at 100 % (m/m) each. Although the maximum input amount of DNA is restricted to certain limits (e.g. 200 ng/reaction), an high DNA amount of the targets in excess can be used (see Table 15 for further calculations)

Target sequence A (copies/reaction)	**Target sequence B (copies/reaction)**	**Target sequence C (copies/reaction)**
25	20,000	20,000
10	20,000	20,000
5	20,000	20,000
3	20,000	20,000
1	20,000	20,000
0.1	20,000	20,000

Table 15 Calculation of the DNA input for Example 1 for the determination of LOD_{asym} of one target in a triplex-PCR method. Here, all target sequences are from heterozygous GM maize with an available reference material at 100 % (m/m) each. Although the maximum input amount of DNA is restricted to certain limits (e.g. 200 ng/reaction), a high DNA amount of the targets in excess can be used

Number of modules	3	-plex
Maximum total DNA input	200	ng/rcn

Target No.	Species	RM % (w/w)	Mass of haploid genome (pg)	Conversion Factor (Zygosity)	Final DNA Concentration		Final GM % (w/w)
					target (cp/rcn)	total (ng/rcn)	
Target 1	maize	100	2.73	0.50	25	0.14	0.07
Target 2	maize	100	2.73	0.50	18,336	99.9	50.0
Target 3	maize	100	2.73	0.50	18,336	99.9	50.0
						Total DNA input 200	

Table 16 Example 2. Determination of LOD_{asym} of one target in a triplex-PCR method (replicates at each level n=12). Here, all target sequences are from heterozygous GM maize with an available reference material at only 5 % (m/m) each. Since the maximum input amount of DNA is restricted to certain limits (e.g. 200 ng/reaction), the maximum amount of the targets in excess is limited (see Table 17 for further calculations)

Target sequence A (copies/reaction)	**Target sequence B (copies/reaction)**	**Target sequence C (copies/reaction)**
50	1,000	1,000
20	1,000	1,000
10	1,000	1,000
5	1,000	1,000
1	1,000	1,000
0.1	1,000	1,000

Table 17 Calculation of the DNA input for Example 2 for the determination of LOD_{asym} of one target in a triplex-PCR method. Here, all target sequences are from heterozygous GM maize with an available reference material at only 5 % (m/m) each. Since the maximum input amount of DNA is restricted to certain limits (e.g. 200 ng/reaction), the maximum amount of the targets in excess is limited

Number of modules	3	-plex
Maximum total DNA input	200	ng/rcn

Target No.	Species	RM % (w/w)	Mass of haploid genome (pg)	Conversion Factor (Zygosity)	Final DNA Concentration		Final GM % (w/w)
					target (cp/rcn)	total (ng/rcn)	
Target 1	maize	5	2.73	0.5	50	5.45	0.12
Target 2	maize	5	2.73	0.5	892	97.3	2.4
Target 3	maize	5	2.73	0.5	892	97.3	2.4
						Total DNA input 200	

Table 18 Example 3. Determination of LOD_{asym} of one target in a tetraplex-PCR method (replicates at each level n=12). Here, all target sequences are from homozygous GM soybean with an available reference material at only 5 % (m/m) each. Since the maximum input amount of DNA is restricted to certain limits (e.g. 200 ng/reaction), the maximum amount of the targets in excess is limited (see Table 19 for further calculations)

Target sequence A (copies/reaction)	**Target sequence B (copies/reaction)**	**Target sequence C (copies/reaction)**	**Target sequence D (copies/reaction)**
20	3,000	3,000	3,000
10	3,000	3,000	3,000
5	3,000	3,000	3,000
2	3,000	3,000	3,000
1	3,000	3,000	3,000
0.1	3,000	3,000	3,000

Table 19 Calculation of the DNA input for Example 3 for the determination of LOD_{asym} of one target in a tetraplex-PCR method. Here, all target sequences are from homozygous GM soybean with an available reference material at only 5 % (m/m) each. Since the maximum input amount of DNA is restricted to certain limits (e.g. 200 ng/reaction), the maximum amount of the targets is limited

Number of modules	4	-plex
Maximum total DNA input	200	ng/rcn

Target No.	Species	RM % (w/w)	Mass of haploid genome (pg)	Conversion Factor (Zygosity)	Final DNA Concentration		Final GM % (w/w)
					target (cp/rcn)	total (ng/rcn)	
Target 1	soybean	5	1.13	1	20	0.5	0.01
Target 2	soybean	5	1.13	1	2943	66.5	1.7
Target 3	soybean	5	1.13	1	2943	66.5	1.7
Target 4	soybean	5	1.13	1	2943	66.5	1.7
					Total DNA input	200	

Table 20 Example 4. Determination of LOD_{asym} of one target in a tetraplex-PCR method (replicates at each level n=12). Here, the target sequences are from GM soybean, maize and rapeseed with an available reference material ranging from 10 % to 100 % (m/m). Since the maximum input amount of DNA is restricted to certain limits (e.g. 200 ng/reaction), the maximum amount of the targets in excess is limited (see Table 21 for further calculations)

Target sequence A (copies/reaction)	**Target sequence B (copies/reaction)**	**Target sequence C (copies/reaction)**	**Target sequence D (copies/reaction)**
20	2,500	2,500	2,500
10	2,500	2,500	2,500
5	2,500	2,500	2,500
2	2,500	2,500	2,500
1	2,500	2,500	2,500
0.1	2,500	2,500	2,500

Table 21 Calculation of the DNA input for Example 4 for the determination of the LOD_{asym} of one target in a tetraplex-PCR method. Here, the target sequences are from GM soybean, maize and rapeseed with an available reference material ranging from 10 % to 100 % (m/m). Since the maximum input amount of DNA is restricted to certain limits (e.g. 200 ng/reaction), the maximum amount of the targets in excess is limited

Number of modules	4	-plex
Maximum total DNA input	200	ng/rcn

Target No.	Species	RM % (w/w)	Mass of haploid genome (pg)	Conversion Factor (Zygosity)	Final DNA Concentration	
					target (cp/rcn)	total (ng/rcn)
Target 1	soybean	10	1.13	1	20	0.5
Target 2	maize	10	2.73	0.5	2400	130.8
Target 3	maize	100	2.73	0.5	2400	13.1
Target 4	rapeseed	10	1.15	0.5	2400	55.2
					Total DNA input	200

Annex C: *In silico* specificity assessment

In order to predict potential false positive signals in multiplexing, all possible combinations of oligonucleotide sequences (primer, probe) should be evaluated in a computer-aided (*in silico*) test for the compatibility between primers and similarities to sequences of other genomes.

Primers and probe compatibility

To verify the compatibility of primers, it may be necessary to analyse different features such as the melting temperature (T_m) of the oligonucleotides, secondary structures and cross dimers between all the primers included in the multiplex test. To achieve this, several software programs can be used. All the programs used to test the compatibility for a single target PCR are suitable.

Table 22 presents an overview of programs with their features. Determining which software is the most appropriate will depend on the exploitation system available, the number of primer pairs to test and the user's skills and familiarity with this type of tool. The analysis will focus mainly on primers, as it seems that probes are less affected by multiplexing for the considered parameters (Lutz Grohmann et al. 2017). It should be stressed that associations of dyes linked to the probes are not taken into account in this analysis. Most testing tools are able to test several couples of primers in a multiplex format. However, the compatibility between primers and probe is generally only available in a singleplex format. It may be necessary to check the compatibility of probes and the presence of potential secondary structures by introducing them as primers in the program. The T_m of the probes has also to be checked. The melting temperature of the probes has to be compatible with those of the primers (the T_m of the probe is generally 8-10 °C higher than the T_m of the primers) and probes used in the same PCR reaction must have a similar T_m.

Checking the specificity by *in silico* PCR

This step will search for possible similarities within a database of DNA sequences to confirm the amplification of the expected sequences and highlight possible unwanted/unexpected amplifications. The database can be a large collection of sequences (e.g. NCBI; see Petrillo *et al.*, 2015) or a restricted database such as the GMO sequences stored in the JRC Central Core Sequence Information (Patak 2011; Ye et al. 2012). Primers and probes have to be tested to ensure their specificity.

It is very important to emphasize the importance of the database being queried. The database has to contain plant genomes, GMO sequences, but also the donor organisms of the structural elements.

The main computer programs used to perform this *in silico* PCR are briefly compared in Table 22.

PrimerBlast is an online tool. This program is easy to use and allows to query directly the collection of sequences stored on NCBI. The major disadvantages of this tool are that it is only available online and that primers can only be tested by couples. This tool is based on Primer3.

ecoPCR is a command line tool. It is less user friendly in comparison to the previous one. This tool was initially foreseen to test primers for molecular barcoding. With a bioinformatics pipeline (not provided) it is possible to automate the testing of each pair of primers. The database queried by ecoPCR has to be previously created with **obiconvert** (Boyer et al. 2016) (see example in Marquina *et al.*, 2019).

MFEprimer is a program allowing *in silico* amplification and checking the formation of inappropriate secondary structures. An interesting option is the possibility to test a set of primers (not only a single couple of primers). When used online, only a limited number of databases are available to check possible amplifications, but once downloaded, it is possible to link the search to more complete collections of sequences gathered in the standard FASTA format. This program is easy to use and includes multiple options.

FastPCR allows *in silico* PCR to be used. As for MFEPrimer, a collection of sequences in the FASTA format has to be provided for comparison.

Note: e-PCR, provided by NCBI, was discontinued and is no longer maintained. Primer-BLAST (Ye et al. 2012) is proposed as an alternative program (see Patak, 2011)

Table 22 Non-exhaustive list of frequently used computer programs for primer compatibility and *in silico* PCR

Programs	Local (L) or Online (O)	Number of primers tested simultaneously	Operating System	Type of interface	Primers Compatibility (structure)	Primers specificity (*in Silico* PCR)	Configurable	Reference
Primer Express[1]	L	2	Windows	GUI*	X		yes	
Multiple primer Analyzer[2]	O	Many	All	Web	X		limited	
FastPCR[3]	O+L	Many	All (online) or windows (local)	GUI*	X	X	yes	(Kalendar *et al.* 2011)
MFEPrimer[4]	O+L	Many	All (online) or IOs, Linux (local)	Web or Command line	X	X	yes	(Qu and Zhang 2015)
PrimerBlast[5]	O	2	All	Web		X	yes	(Ye et al. 2012)
ecoPCR	L	2 but automation possible	IOs, Linux	Command line		X	yes	(Bellemain et al. 2010)
Primer3	O+L	2 (online), Many with local software	All	Web or Command line	X		yes	

*Graphical User Interface

[1] Primer Express® Software v3.0.1 Applied Biosystems by Thermo Fischer Scientific (with licence)
[2]Multiple-Primer-analyser Thermo Fischer Scientific: https://www.thermofisher.com/be/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html
[3] FastPCR Primerdigital: http://primerdigital.com/fastpcr.html
[4] MFEprimer: https://www.mfeprimer.com/
[5] Primer Blast: https://www.ncbi.nlm.nih.gov/tools/primer-blast

Annex D: Examples of practical settings for robustness testing

The experimental design for robustness testing allows any pair of factors (e.g. annealing temperature and primer concentration of each individual module) to be represented at least twice in all possible factor combinations (1/1, 1/2, 2/1 and 2/2). Thus, the design will also detect possible factorial interactions. With the multi-factorial study design approach, the testing of many factors with reduced effort is achievable.
Examples of multi-factorial design approaches are provided below.

Table 23 Multifactorial design of the conditions outlined in Table 10 during robustness assessment of a duplex PCR method

Factor	**Combination**							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Annealing temperature	1	1	1	1	2	2	2	2
Reaction volume	1	1	2	2	1	1	2	2
primer concentration module 1	1	1	2	2	2	2	1	1
probe concentration module 1	1	2	1	2	1	2	1	2
primer concentration module 2	1	2	1	2	2	1	2	1
probe concentration module 2	1	2	2	1	1	2	2	1
Master mix concentration	1	2	2	1	2	1	1	2

Figure 3 Example of PCR plate setup for multifactorial design of the conditions outlined in Table 23 during robustness assessment of a quantitative duplex PCR method (i.e. GM assay and taxon-specific assay). Since the taxon-specific target (B) is present in excess, only the GM target sequence (A) has to be tested at the LOQ_{asym}. One standard curve under optimal (unchanged) conditions except the annealing temperature should be used per PCR plate.

Plate A (- 1 °C Annealing Temperature)

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	Combination 1			Combination 2			Combination 3			Combination 4		
B												
C	Unchanged Conditions (except Annealing Temperature)											
D												
E	Combination 5		Combination 6		Combination 7		Combination 8					
F												
G												
H												

Plate B (+ 1 °C Annealing Temperature)

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	Combination 5			Combination 6			Combination 7			Combination 8		
B												
C	Unchanged Conditions (except Annealing Temperature)											
D												
E	Combination 5		Combination 6		Combination 7		Combination 8					
F												
G												
H												

Target Sequence A at LOQ_{asym} (Sequence B in excess, n = 3)
Standard curve (5 calibrations points, n = 2)
NTC (No Template Control, n = 2)

Table 24 Multifactorial design of the conditions outlined in Table 8 during robustness assessment of a tetraplex PCR method

Factor	Combination											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Annealing temperature	1	1	1	1	1	1	2	2	2	2	2	2
Reaction volume	1	1	1	2	2	2	1	1	1	2	2	2
primer conc. module 1	1	1	2	1	2	2	2	2	1	2	1	1
probe conc. module 1	1	1	2	2	1	2	2	1	2	1	2	1
primer conc. module 2	1	1	2	2	2	1	1	2	2	1	1	2
probe conc. module 2	1	2	1	1	2	2	1	2	2	1	2	1
primer conc. module 3	1	2	1	2	1	2	2	2	1	1	1	2
probe conc. module 3	1	2	1	2	2	1	2	1	2	2	1	1
primer conc. module 4	1	2	2	1	1	2	1	1	2	2	1	2
probe conc. module 4	1	2	2	1	2	1	2	1	1	1	2	2
Master mix concentration	1	2	2	2	1	1	1	2	1	2	2	1

Figure 4 Example of a PCR plate setup for the multifactorial design of the conditions outlined in Table 20 during robustness assessment of a qualitative tetraplex PCR method

Plate A (- 1 °C Annealing Temperature)

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	Combination 1			Combination 2			Combination 3			Combination 4		
B	Combination 5			Combination 6			Combination 1			Combination 2		
C	Combination 3			Combination 4			Combination 5			Combination 6		
D	Combination 1			Combination 2			Combination 3			Combination 4		
E	Combination 5			Combination 6			Combination 1			Combination 2		
F	Combination 3			Combination 4			Combination 5			Combination 6		
G												
H	Combination 1		Combination 2		Combination 3		Combination 4		Combination 5		Combination 6	

Plate B (+ 1 °C Annealing Temperature)

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	Combination 7			Combination 8			Combination 9			Combination 10		
B	Combination 11			Combination 12			Combination 7			Combination 8		
C	Combination 9			Combination 10			Combination 11			Combination 12		
D	Combination 7			Combination 8			Combination 9			Combination 10		
E	Combination 11			Combination 12			Combination 7			Combination 8		
F	Combination 9			Combination 10			Combination 11			Combination 12		
G												
H	Combination 7		Combination 8		Combination 9		Combination 10		Combination 11		Combination 12	

Target Sequence A at LOD_{asym} (other in excess)

Target Sequence B at LOD_{asym} (other in excess)

Target Sequence C at LOD_{asym} (other in excess)

Target Sequence D at LOD_{asym} (other in excess)

NTC (No Template Control)

Annex E: Summary of practical settings for in house validation and verification

Table 25 Overview of the tests to be carried out for single-laboratory validation of qualitative mpPCR methods

Performance parameter	Material	PCR replicates	Dilution levels	Copies of target sequence per PCR mix	Comments
Limit of detection					
LOD_{abs} (optional)	Target DNA[1] in background DNA[2]	12 per module	e.g. 6	e.g. 50, 25, 10, 5, 1, 0.1	The target sequences of all modules are checked with one calibration solution adjusted to uniform copy numbers.
LOD_{asym}	Target DNA[1] of all modules (asymmetric conditions)	12 per module	e.g. 6	e.g. 50, 25, 10, 5, 1, 0.1 in excess of the other target sequences (≥ 2,500 copies)	The LOD_{asym} for each individual module should be < 25 copies/reaction in excess of other target(s). The amount of other target(s) under asymmetric conditions depends on the scope of the multiplex method.
Specificity					
Theoretical test (*in silico*)	Sequence databases (e.g. BLAST in GenBank, web service „JRC GMO-Amplicons")	-	-	-	Testing of relevant plant/animal species or GMO. Testing of all primer and probe combinations for similarities to other sequences, complementarity/formation of artefacts.
Practical test	Target DNA[1] in background DNA[2]; non-target DNA[3]	2	-	Target DNA[1]: 100; non-target DNA[3]: 2,500	If no material[3] with a sufficiently high concentration is available, fewer copies can be used. Testing for additional amplicons or artefacts by means of gel or capillary electrophoresis or, if applicable, a melting curve analysis of the PCR products.
Cross-talk	Target DNA[1]	3 per module	-	0 copies of the module to be tested in excess (e.g. 20,000 copies) of the other target sequence(s)	Minimal cross-talk might be acceptable. Improvement can be reached by reducing the concentration of the 'cross-talking' probe or by changing the probe label.
Robustness	Target DNA[1] of all modules (asymmetric conditions)	3 per condition	-	At LOD_{asym} (e.g. 20 in excess of the other target sequences)	Annealing temperature, reaction volume and master mix and oligonucleotide concentrations should be varied (several factors are not amendable because of technical reasons). Multi-factorial study design reduces effort.

1 = genomic DNA, plasmid DNA or amplicon DNA;

2 = DNA to stabilize the PCR e.g. plant species or salmon sperm DNA in a concentration of up to 100 – 200 ng per 25 µL of PCR mix;

3 = DNA from GMO containing similar genetic elements or constructs, as well as DNA from plant or animal species frequently to be found in samples.

Table 26 Overview of the tests to be carried out for verification of qualitative mpPCR methods

Performance parameter	Material	PCR replicates	Dilution levels	Copies of target sequence per PCR mix	Comments
Limit of detection					
LOD_{abs} (optional)	Target DNA[1] in background DNA[2]	10 per module	e.g. 6	According to the validation report e.g. 50, 25, 10, 5, 1, 0.1	The target sequences of all modules are checked with one calibration solution adjusted to uniform copy numbers.
LOD_{asym}	Target DNA[1] of all modules (asymmetric conditions)	10 per module	e.g. 6	According to the validation report e.g. 50, 25, 10, 5, 1, 0.1 in excess of the other target sequences (≥ 2,500 copies)	The LOD_{asym} for each individual module should be < 25 copies/reaction in excess of other target(s). The amount of other target(s) under asymmetric conditions depends on the scope of the multiplex method.
Cross-talk	Target DNA[1]	3 per module	-	0 copies of the module to be tested in excess (e.g. 20,000 copies) of the other target sequence(s)	Minimal cross-talk might be acceptable. Improvement can be reached by reducing the concentration of the 'cross-talking' probe or by changing the probe label.

1 = genomic DNA, plasmid DNA or amplicon DNA;

2 = DNA to stabilize the PCR e.g. plant species or salmon sperm DNA in a concentration of up to 100 – 200 ng per 25 µL of PCR mix;

3 = DNA from GMO containing similar genetic elements or constructs, as well as DNA from plant or animal species frequently to be found in samples.

Table 27 Overview of the tests to be carried out for single-laboratory validation of quantitative duplex real-time PCR methods (i.e. GM assay and taxon-specific assay)

Performance parameter	Material	PCR replicates	Dilution levels	Copies of target sequence per PCR mix	Comments
Limit of quantification					
LOQ_{abs} (optional)	Target DNA[1] in background DNA[2]	15 per module	e.g. 4	e.g. 50, 40, 20, 10	The target sequences of both modules are checked with one calibration solution adjusted to uniform copy numbers.
LOQ_{asym}	Target DNA[1] of both modules (asymmetric conditions)	15 per module	e.g. 4	GM gene: e.g. 50, 40, 20, 10 in excess of taxon-specific target: ≥ 56,000	The LOQ_{asym} for each individual module should be ≤ 50 copies/reaction in excess of the other target. The amount of other target under asymmetric conditions depends on the scope of the multiplex method.
Dynamic range, R^2 coefficient & amplification efficiency	Target DNA[1] of both modules (asymmetric conditions)	3	5 (5 curves in total)	GM gene: 50-2,520 in excess of taxon-specific target: 50-56,000	Individual values of at least five independent standard curves should be considered. All slopes shall be in the range of -3.6 ≤ slope ≤ -3.1 and all R^2 values should be ≥ 0.98. Asymmetric ratio of 1/10 for the calibration material is recommended. Validated simultaneously from standard curves when testing trueness and precision.
Trueness & Precision	Target DNA[1] of both modules (asymmetric conditions, e.g. CRM)	15 (intermed. precision conditions)	3	Copies in the dynamic range corresponding to e.g. 0.09 % m/m, 0.9 % m/m and 4.5 % m/m	The trueness should be within ± 25 % of the accepted reference value. The RSD_r should be ≤ 25 % over the dynamic range of the method.
Specificity					
Theoretical test (*in silico*)	Sequence databases (e.g. BLAST in GenBank, web service „JRC GMO-Amplicons")	-	-	-	Testing of relevant plant/animal species or GMO. Testing of all primer and probe combinations for similarities to other sequences, complementarity/formation of artefacts.
Practical test	Target DNA[1] in background DNA[2]; non-target DNA[3]	2	-	Target DNA[1]: 100; non-target DNA[3]: 2,500	If no material[3] with a sufficiently high concentration is available, fewer copies can be used. Testing for additional amplificons or artefacts by means of gel or capillary electrophoresis or, if applicable, a melting curve analysis of the PCR products.

Cross-talk	Target DNA[1]	3 per module	-	GM gene: 0 copies in excess of taxon-specific target: ≥ 56,000	Minimal cross-talk might be acceptable. Improvement can be reached by reducing the concentration of the 'cross-talking' probe or by changing the probe label.
Robustness	Target DNA[1] of all modules (asymmetric conditions)	3 per condition	-	At LOQ_{asym} (i.e. GM gene: ≤ 50 copies in excess of taxon-specific target: ≥ 56,000)	Annealing temperature, reaction volume and master mix and oligonucleotide concentrations should be varied (several factors are not amendable because of technical reasons). Multi-factorial study design reduces effort.

1 = genomic DNA, plasmid DNA or amplicon DNA;

2 = DNA to stabilize the PCR e.g. plant species or salmon sperm DNA in a concentration of up to 100 – 200 ng per 25 µL of PCR mix;

3 = DNA from GMO containing similar genetic elements or constructs, as well as DNA from plant or animal species frequently to be found in samples.

Table 28 Overview of the tests to be carried out for verification of quantitative duplex real-time PCR methods (i.e. GM assay and taxon-specific assay)

Performance parameter	Material	PCR replicates	Dilution levels	Copies of target sequence per PCR mix	Comments
Limit of quantification					
LOQ_{abs} (optional)	Target DNA[1] in background DNA[2]	10 per module	e.g. 1	According to the validation report e.g. 50	The target sequences of both modules are checked with one calibration solution adjusted to uniform copy numbers.
LOQ_{asym}	Target DNA[1] of both modules (asymmetric conditions)	10 per module	e.g. 1	According to the validation report e.g. GM gene: 50 in excess of taxon-specific target: ≥ 56,000	The LOQ_{asym} for each individual module should be ≤ 50 copies/reaction in excess of the other target. The amount of other target(s) under asymmetric conditions depends on the scope of the multiplex method.
Dynamic range, R^2 coefficient & amplification efficiency	Target DNA[1] of both modules (asymmetric conditions)	2	5 (2 curves in total)	According to the validation report e.g. GM gene: 50-2,520 in excess of taxon-specific target: 50-56,000	Individual values of at least two independent standard curves should be considered. All slopes shall be in the range of -3.6 ≤ slope ≤ 3.1 and all R^2 values should be ≥ 0.98. Validated simultaneously from standard curves when testing trueness and precision.
Trueness & Precision	Target DNA[1] of both modules (asymmetric conditions, e.g. CRM)	15	2	Copies in the dynamic range corresponding to e.g. 0.09 % m/m, 0.9 % m/m and 4.5 % m/m (optional)	The trueness should be within ± 25 % of the accepted reference value. The RSD_r should be ≤ 25 %, over the dynamic range of the method.
Cross-talk	Target DNA[1]	3 per module	-	GM gene: 0 copies in excess of taxon-specific target: ≥ 56,000 copies	Minimal cross-talk might be acceptable. Improvement can be reached by reducing the concentration of the 'cross-talking' probe or by changing the probe label.

1 = genomic DNA, plasmid DNA or amplicon DNA;

2 = DNA to stabilize the PCR e.g. plant species or salmon sperm DNA in a concentration of up to 100 – 200 ng per 25 µL of PCR mix;

3 = DNA from GMO containing similar genetic elements or constructs, as well as DNA from plant or animal species frequently to be found in samples.

Annex F: Method collection

Table 29 Examples of available mpPCR methods for GMO detection (by 01.10.2020)

Application	Targets	Type of PCR and Multiplex level	Validation status	Reference
screening for GMOs containing P-35S, T-nos	P-35S, T-nos	real-time 2-plex	interlaboratory	Waiblinger *et al.*, 2008
screening for GM events without screening elements and that are expected negative	GM soybean events: MON 87701, MON 87708, MON 87769, DP-305423, CV-127, DAS-68416	real-time 6-plex single colour	interlaboratory	Grohmann *et al.*, 2014
screening for GM crops containing pat, bar	pat, bar	real-time 2 -plex	interlaboratory	Debode *et al.*, 2017
all GM crops containing one or more of the screening elements/construct	P-35S, T-nos, pat, bar, ctp2/cp4-epsps	real-time 2-plex, 3-plex, 5-plex	interlaboratory	Huber *et al.*, 2013
screening for GMOs containing P-35S, T-nos, P-FMV, T-35S	P-35S, T-nos, P-FMV, T-35S	real-time 4-plex	interlaboratory	Eugster *et al.*, 2014
quantification of 3 rice GMO events and housekeeping gene and 35S/Bar	3 GM rice events	real-time 5-plex	in-house	Köppel, Zimmerli and Breitenmoser, 2010
quantification of 4 soy GMO events and housekeeping gene	4 GM soybean events	real-time 5-plex	in-house	Köppel *et al.*, 2012
quantification of 4 soy GMO events and housekeeping gene	4 GM soybean events	real-time 5-plex	in-house	Köppel *et al.*, 2015
all GM crops containing one or more of the screening elements/construct	P-35S, P-FMV, T-nos, hmg, lectin, pat, bar, ctp2/cp4-epsps, CaMV	real-time 4 and 5-plex	in-house	Köppel *et al.,* 2014

Application	Targets	Type of PCR and Multiplex level	Validation status	Reference
quantification of 5x4 maize GMO events and housekeeping gene	quantification of 20 maize GMO events and housekeeping gene	real-time 5-plex	in-house	Köppel *et al.*, 2017
screening for GM elements and events	1 plant, 8 endogenous genes, P-35S, T-nos, bar, pat, nptII, P-FMV, T-E9, cp4-epsps, Hph, cry1Ab/c, 26 events, KMD1 construct, Xa21 gene	real-time 2-plex	in-house	Cottenet *et al.*, 2013
GTS 30-4-2 soybean event quantification	lectin, cp4-epsps/T-nos event specific border	real-time 2-plex	in-house	Samson *et al.,* 2010
GTS 30-4-2 soybean construct quantification	lectin, ctp4/cp4-epsps construct	real-time 2-plex	in-house	Foti *et al.*, 2006
quantification of several GM maize events	12 GM maize events	digital 4-plex, 10-plex	in-house	Dobnik *et al.*, 2015

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