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Overview and recommendations for the application of digital PCR

European Network of GMO Laboratories (ENGL)

Pecoraro S., Berben G., Burns M., Corbisier P., De Giacomo M., De Loose M., Dagand E., Dobnik D., Eriksson R., Holst-Jensen A., Kagkli D. M., Kreysa J., Lievens A., Mäde D., Mazzara M., Paternò A., Peterseil V., Savini C., Sovová T., Sowa S., Spilsberg B.

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Contact information
ENGL Secretariat
Food & Feed Compliance Unit (F.5)
European Commission - Joint Research Centre (JRC)
Health, Consumers and Reference Materials
Via Enrico Fermi 2749. TP 201
I-21027 Ispra (VA) Italy

E-mail: JRC-ENGL-SECRETARIAT@ec.europa.eu

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Contents

1. Executive summary 3
2. Working group overview 4
2.1 Working group establishment 4
2.2 Background 4
2.3 Mandate and tasks 4
2.4 Scope 5
3. Glossary 5
4. Introduction and description of technologies 7
4.1 Introduction 7
4.1.1 Chamber-based methods 10
4.1.2 Droplet-based methods 10
4.2 Fields of application 10
4.2.1 Application of dPCR in the field of GMO detection and quantification 10
4.2.2 Digital PCR applications for monitoring human disease states 13
4.2.3 Digital PCR applications in the field of food virology 14
4.2.4 Digital PCR in the agricultural and environmental field 14
4.2.5 Digital PCR applications in the field of species identification 15
4.2.6 Digital PCR applications in the field of microbiology 15
5. Technical aspects of digital PCR 16
5.1 Theoretical basis of digital PCR 16
5.1.1 Statistics and assumptions 16
5.2 DNA extraction 18
5.2.1 Sample preparation 18
5.2.2 DNA yield 18
5.2.3 DNA structural integrity 18
5.2.4 Purity of DNA extracts 19
5.2.5 Additional recommendations 19
5.3 PCR 20
5.3.1 Master mix 20
5.3.2 Primers and probe concentrations 21
5.3.3 Temperature cycling program 22

5.3.4 The use of control samples 25
5.3.5 Plasticware 25
5.3.6 Considerations for multiplexing 25
5.4 Results and performance requirements 27
5.4.1 Amplification product of a single target 28
5.4.2 Amount of rain 29
5.4.3 Resolution 29
5.4.4 Target concentration 30
5.4.5 Amount of sample compartmentalized 31
5.4.6 Unit of measurement 31
5.5 Data interpretation and reporting 32
5.5.1 Data interpretation 32
5.6 Digital MIQE guidelines 34
6. Costs and practicability of dPCR 35
6.1 Instruments costs 35
6.2 Consumables cost 35
6.3 Practicability 36
6.4 In-house verification 37
6.4.1 Specificity 37
6.4.2 Repeatability 38
6.4.3 Intermediate precision 38
6.4.5 Trueness 39
6.4.6 Uncertainty estimation 40
6.4.7 Limit of Detection (LOD), Limit of Quantification (LOQ) 41
6.4.8 Robustness 41
6.5 Accreditation 42
7. Bibliography 43
Annex 50
A. Application of dPCR in the field of GMO detection and quantification – explanations, notes and examples 50
B. Technical part specific for the implementation of dPCR in a laboratory 53

1. Executive summary

The so-called digital Polymerase Chain Reaction (dPCR) is a relatively new technique for the detection and quantification of DNA, but its application in analytical laboratories is steadily increasing. In contrast to quantitative real-time PCR, DNA (fragments) can be quantified here without the need for calibration curves. Using dPCR, the PCR mix containing the (target) DNA is partitioned – depending on the device used – currently into a maximum of 10,000,000 small compartments with a volume as low as a few picolitres. These can be either physically distinct compartments on a chip (referred to as chamber-based digital PCR [cdPCR]), or the compartments correspond to water-in-oil droplets (referred to as droplet digital [ddPCR]). Once the PCR has been carried out simultaneously in all compartments/droplets, it is common to both approaches that the number of positive and negative signals for each partition is counted by a fluorescence measurement.

With this technique, an absolute quantification of DNA copy numbers can be performed with high precision and trueness, even for very low DNA copy numbers. Furthermore, dPCR is considered less susceptible than qPCR to PCR inhibitory substances that could be co-extracted during DNA extraction from different samples.

Digital PCR has already been applied in various fields, for example for the detection and quantification of GMOs, species (animals, plants), human disease bioindicators, food viruses and bacteria including pathogens.

When establishing dPCR in a laboratory, different aspects have to be considered. These include, but are not limited to, the adjustment of the type of the PCR master mix used, optimised primer and probe concentrations and the signal separation of positive and negative compartments. This document addresses these and other aspects and provides recommendations for the transfer of existing real-time PCR methods into a dPCR format.

2. Working group overview

2.1 Working group establishment

The Working Group (WG) on digital PCR was established based on a mandate adopted at the 28th meeting of the ENGL (European Network of GMO Laboratories) Steering Committee on 11th February 2015. The WG was chaired by Sven Pecoraro, Bavarian Health and Food Safety Authority (LGL), Oberschleissheim, Germany.

The other members of the Working Group were: Gilbert Berben, Walloon agricultural Research Center (CRA-W), Belgium; Malcolm Burns, LGC, United Kingdom; Philippe Corbisier, European Commission, Directorate-General Joint Research Centre (DG JRC), Belgium; Marzia De Giacomo, Italian Institute of Health (ISS), Italy; Marc De Loose, Flanders research institute for agriculture, fisheries and food (ILVO), Belgium; Emilie Dagand, Federal Office of Consumer Protection and Food Safety (BVL), Germany; David Dobnik, National Institute of Biology (NIB), Slovenia; Ronnie Eriksson, National Food Agency (NFA), Sweden; Arne Holst-Jensen, Norwegian Veterinary Institute (NVI), Norway; Dafni-Maria Kagkli, European Commission, Directorate-General Joint Research Centre (DG JRC), Italy; Joachim Kreysa, European Commission, Directorate-General Joint Research Centre (DG JRC), Belgium; Antoon Lievens, European Commission, Directorate-General Joint Research Centre (DG JRC), Belgium; Dietrich Mäde, State Institute for Consumer Protection Department of Food Safety Saxony-Anhalt (LAV ST), Germany; Marco Mazzara, European Commission, Directorate-General Joint Research Centre (DG JRC), Italy; Annalisa Paternò, Istituto Zooprofilattico Sperimentale del Lazio e della Toscana- M. Aleandri (IZSLT), Italy; Verena Peterseil, Austrian Agency for Health and Food Safety (AGES), Austria; Christian Savini, European Commission, Directorate-General Joint Research Centre (DG JRC), Italy; Tereza Sovová, Crop Research Institute (CRI), Czech Republic; Slawomir Sowa, Plant Breeding and Acclimatization Institute (IHAR) - National Research Institute, Poland; Bjørn Spilsberg, Norwegian Veterinary Institute (NVI), Norway.

2.2 Background

Digital PCR (dPCR), in its different formats (chamber dPCR, droplet dPCR), is a rapidly evolving technology in the area of DNA analysis. Digital PCR brings various advantages over traditional real-time PCR, including the large number of parallel repetitions (from a few hundred to thousands per sample), the potential to conduct absolute quantification without standard curves, and the reduced sensitivity to PCR inhibitors affecting DNA analysis.

During 2014, the ENGL discussed the current application of dPCR to GMO analysis and identified that the technology had the potential to advance DNA analysis applied in a regulatory context. Advantages and disadvantages were identified during an ENGL discussion day where experts also identified some issues to be resolved to facilitate routine application of dPCR for DNA analysis.

2.3 Mandate and tasks

As part of the mandate from the ENGL Steering Committee, the WG was asked to review the following issues, identify future needs and propose approaches to address these:

- Transferability of existing real-time PCR methods into a dPCR format;
- Accreditation (including in-house validation);
- Applicability to difficult matrices;
- Applicability to analytical areas other than GM food/feed;

- Definition and assessment of relevant method performance criteria;
- Multiplexing;
- Summary of technical needs and requirements for implementing and applying dPCR.

As a result, the following document was elaborated, addressing the various issues discussed and summarising relevant existing experience with dPCR, with the aim of helping laboratories to decide if dPCR will meet their specific needs.

2.4 Scope

The scope of the document is two-fold: firstly, the potential of the dPCR technologies present on the market and a comparison with real-time PCR are discussed. Furthermore, the different fields of application of dPCR are reflected upon. Secondly, it addresses technical issues, which are of relevance to laboratories using the dPCR technology or considering implementing it, and the scope includes issues linked to the method verification and implementation process. In addition, performance parameters relevant to the dPCR are discussed.

3. Glossary

- CB : confidence bounds
- cdPCR : chamber digital PCR
- CNV : copy number variation
- ddPCR : droplet digital PCR
- dMIQE : minimum information for publication of quantitative digital PCR experiments
- DNA : deoxyribonucleic acid
- dPCR : digital PCR
- ENGL : European Network of GMO Laboratories
- EURL GMFF : European Union Reference Laboratory for GM Food & Feed
- GM event : a specific genetic modification (often used as synonym of GMO)
- GM(O) : genetically modified (organism)
- HGE : haploid genome equivalent
- ISO : International Organization of Standardization
- LC/MS : liquid chromatography/mass spectrometry
- LOD : limit of detection
- LOQ : limit of quantification
- MPR : minimum performance requirements
- MRPL : minimum required performance limit
- NGS : next generation sequencing
- PAL : precautionary allergen labelling
- PCR : polymerase chain reaction
- qMIQE : minimum information for publication of quantitative PCR experiments
- qPCR : quantitative real-time PCR
- R_S : resolution of a digital assay
- RSD : relative standard deviation
- RSDr: repeatability standard deviation
- RT-PCR : reverse transcription-polymerase chain reaction
- UGM : unauthorised GMO
- WG : working group
- WG DIR : ENGL working group on 'Detection, Interpretation and Reporting on the presence of authorised and unauthorised genetically modified materials'

4. Introduction and description of technologies

4.1 Introduction

Methods for nucleic acid analysis that are already established and used on a routine basis include the Polymerase Chain Reaction (PCR). PCR came into common use in the 1980s due to the availability of the thermostable *Taq* DNA polymerase, and this allowed for unprecedented amplification and detection of specific DNA sequences. The amplified DNA was often visualized by staining, for example using ethidium bromide on an agarose gel, or by using capillary electrophoresis in a closed and automated instrument. Whilst end-point PCR was a very useful and flexible tool for the detection of specific DNA targets, its main limitation was its qualitative nature (limited to assessing presence or absence but not quantification of the target).

Quantitative real-time PCR (qPCR) allows analysis of the kinetics of the amplification reaction (Higuchi *et al.*, 1992) through monitoring in real-time a fluorescent signal which is directly proportional to the amount of DNA target sequence being generated during PCR amplification. The signal is produced either by fluorescent probes (Rasmussen *et al.*, 1998) or by fluorescent intercalating reagents (Morrison *et al.*, 1998). Quantification (i.e. determination of the amount of target copies present expressed as an *absolute* number) can be achieved during the exponential growth phase of the PCR, provided that an appropriate calibration curve based on standards of known analyte concentration is included in the PCR setup. Relative quantification, i.e. change relative to a reference, can also be achieved and does not require use of standard curves. Moreover, the added selectivity and specificity attributed to the reaction by the presence of the probe makes qPCR one of the most reliable methods and endorses it as the main method used for nucleic acid identification and quantification.

Real-time PCR can suffer from a number of issues including:

- Initial amplification cycles are assumed to be exponential but in practice this may not be the case;
- Low initial concentrations of nucleic acid molecules may not amplify to detectable levels due to the presence of inhibitors; and
- Quantification is relative to a calibration curve (PCR amplification efficiency in a sample of interest may be different from that of reference samples due to matrix differences).

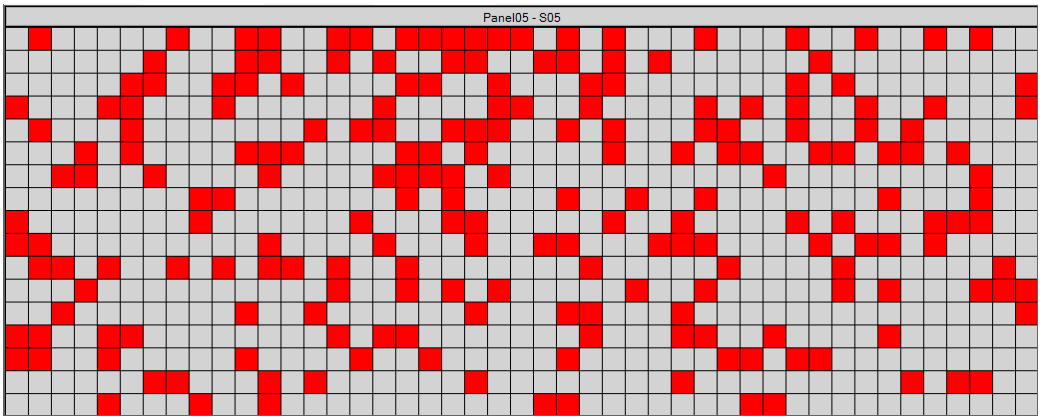
Some of these issues can be minimised or even mitigated entirely using digital PCR (dPCR).

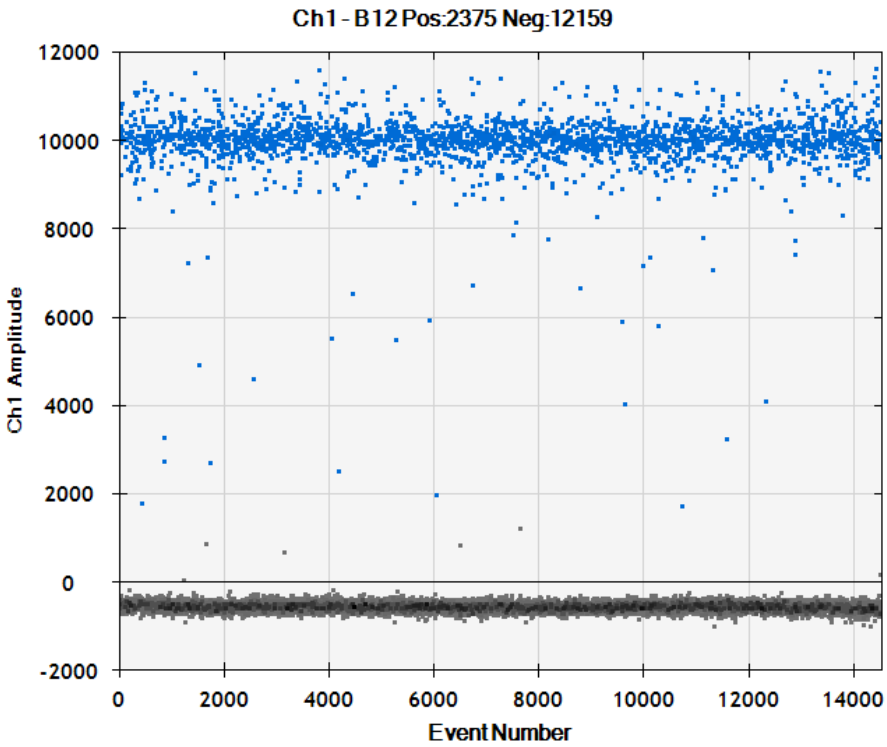
Digital PCR is also based on PCR, but the main difference from qPCR is the fact that the reaction volume is split over a high number of small partitions (from 500 up to millions) of a very small volume (currently from 6 nanoliters down to a few picoliters). After the PCR, each partition is scored either as positive or negative (binary or digital read-out). Statistical analysis of the results is then used to determine the absolute quantity of target DNA in a sample. The approach was first developed in 1999 (Vogelstein and Kinzler, 1999) as a tool for cancer diagnostics, and the underlying principle is well developed in the fields of chemistry and physics, as well as in microbiology where the related most probable number (MPN) method is used to estimate the concentration of specific microorganisms in a matrix (Oblinger and Koburger 1975).

In dPCR, the distribution of target DNA templates throughout the partitions is assumed to follow a Poisson process. Based on counting the total number of positive and negative partitions and using Poisson statistics, the absolute number of DNA copies in the original sample can therefore be estimated (see 4. of this document for a more detailed statistical background).

An example is illustrated in Figure 1. It should be stressed, however, that estimating the number of targets based on a Poisson (or binomial) distribution is based on the following series of assumptions:

- Target molecules are randomly distributed over the total number of partitions under analysis;
- Presence of the target leads to a positive classification of the partition;
- Absence of the target leads to a negative classification of the partition;
- All partitions have the same volume;
- For absolute quantification the volume of the partitions should be known precisely (as the correctness of the measurement depends also on the accuracy with which the partition volume has been determined)





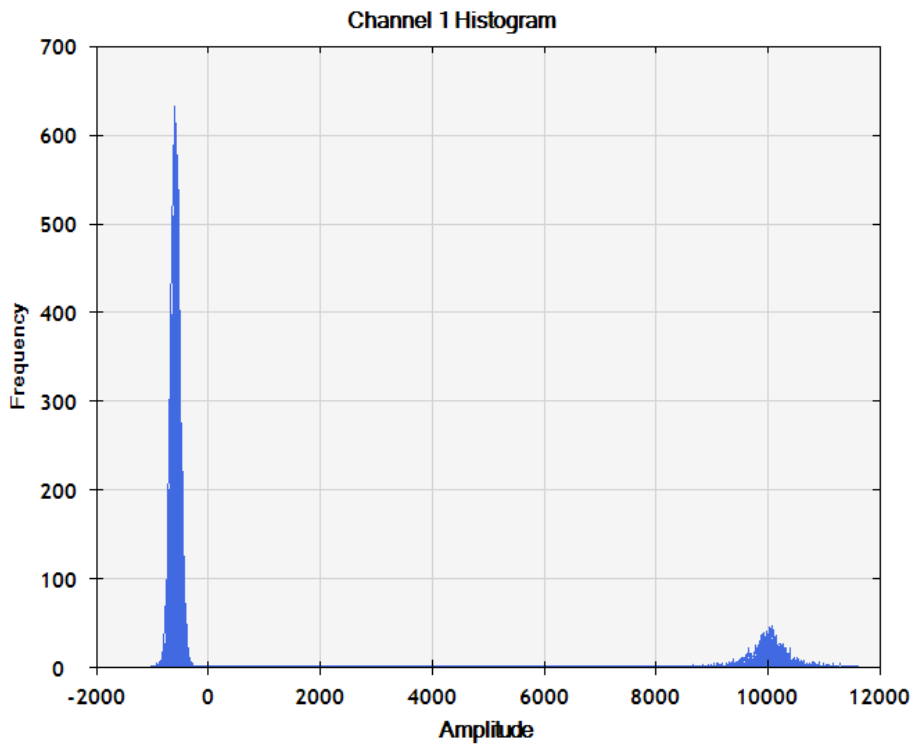


Figure 1: Examples of the results of dPCR runs. The top picture shows an example of the visual output on a Fluidigm dPCR device (cdPCR). The plate (chip) contains 765 partitions, i.e. chambers, of which 208 are shown as positive. The bottom diagram shows an example of the visual output on a Bio-Rad QX200 platform (ddPCR). The reaction contains up to 20,000 droplets, and positive droplets have a higher fluorescence than negative droplets (In the example shown, there are 14,534 droplets in total, of which 2,375 are positive).

Digital PCR possesses a number of advantages compared to conventional endpoint PCR and qPCR:

- The major advantage of the dPCR method is that it permits absolute target quantification without reference to a calibration curve. As a consequence, any matrix differences between calibrant and test sample that may cause different PCR amplification efficiencies are minimised;
- Because of the very high level of sample partitioning achieved (through the high number of individual partitions), dPCR can produce results with very high precision (Hindson *et al.*, 2011);
- Digital PCR may be ideally suited for the detection of minority targets in a high background of competing non-target DNA because in each partition containing the target, the ratio between target and non-target DNA is significantly higher than in the original sample;
- Digital PCR amplification (and consequently the results) are less affected by partial inhibition (Rački *et al*., 2014b; Nixon *et al*., 2014; Iwobi *et al*. 2016).

There are a number of dPCR instruments currently on the market providing evidence of the importance of this relatively new technology for quantitative molecular biology approaches. Digital PCR instruments currently available include "closed" (Fluidigm BioMark, Formulatrix Constellation) or "open" (Thermo Fisher Quant Studio 3D) chamber-based dPCR instruments (for details refer to Basu, 2017) and water-in-oil emulsion droplet-based digital PCR (ddPCR; e.g. Bio-Rad/Bio-Rad: QX, RainDance: RainDrop, Stilla Technologies: Naica System). Future and next generation dPCR devices may include centrifugal devices and sliding microarray devices.

Currently, there are two main approaches for conducting dPCR: *chamber-based methods* and *droplet-based methods*.

4.1.1 Chamber-based methods

Chamber-based methods use pre-made solid-state partitions (chambers) into which the reaction mixture is injected. As with qPCR 96-well plates, the chambers are non-reusable plastic consumables. A dedicated thermal cycler allows the chambers to be cycled and read. The number and size of chambers per device is fixed and thus highly consistent over runs. The number of partitions and reactions per run is often lower than in droplet-based platforms**.**

4.1.2 Droplet-based methods

In ddPCR, the compartmentalization of the reaction mix is achieved by making a water-in-oil emulsion prior to the PCR - generation of high numbers of droplets. The DNA targets in the emulsion are amplified either in standard PCR wells, in strips or on plates and a dedicated reader measures the end-point fluorescence of the droplets. The number of partitions varies between different platforms and between individual reactions.

4.2 Fields of application

Apart from the analysis of Genetically Modified Organisms (GMOs), dPCR has broad applicability across a number of sectors. These include:

- Food fraud and food authenticity testing in general (e.g. meat speciation and quantitation; Floren *et al*., 2015);
- Prenatal diagnostics (e.g. aneuploidy and testing for Down's syndrome; Evans *et al*., 2012);
- Cancer diagnostics (trace detection for a minority target, where early detection means faster treatment and greater chance of successful recovery; Ma *et al*., 2013);
- Viral and bacterial load measurements (important in monitoring and diagnosis of infectious diseases, for example influenza and *Mycobacterium tuberculosis*; Sedlak and Jerome, 2013; and
- Testing for antimicrobial resistance and harmful micro-organisms (Whale *et al*., 2016b).

In the following paragraphs, we give a short overview of how dPCR can be applied in these fields.

4.2.1 Application of dPCR in the field of GMO detection and quantification

GMO detection and quantification – general considerations

For many plant species, e.g. maize, rapeseed, rice and soybean, there are multiple GM events. For GMO detection and quantification, it is common to relate the GM target to a species specific reference target. The reference target can serve multiple functions. The primary function of the reference gene is to inform the analyst of the presence of a particular species (ingredient). Furthermore, if quantified, it can:

- Provide an estimate of the amount of species DNA that is extracted from the sample;
- Provide data on the amount of the species DNA that is necessary if the analyst wishes to quantify the GMO content relative to the species;
- Allow the analyst to determine the practical limit of detection (LOD) and the practical limit of quantification (LOQ) of GM events of the species in question in the sample.

With multiple GM events belonging to the same species, there are several approaches to cost-efficient detection and quantification. Many of the GM events have been transformed using the

same or related genetic constructs resulting in identical or very similar sequences of the inserts of these GMOs. This has been exploited in many routine laboratories applying the so-called element screening (or the matrix approach) for GMO detection as discussed broadly in e.g. Holst-Jensen *et al*. (2012). However, in some cases it may be necessary to help supplement this approach with additional event-specific methods in order to facilitate full coverage of all authorised GM events for a particular species. The results of the screening can be matched against a reference matrix (listing presence/absence data for specific screening markers in all known GMOs). Correspondence between the observed presence/absence patterns and the presence/absence patterns of specific GMOs in the reference matrix suggest possible presence of the specific GMOs in the tested product. Absence of signals for certain elements is indicative evidence of the absence of specific GMOs in the tested products (at the LOD). This information can then be used to select more specific PCRs (construct and event specific real-time PCRs) for identification and quantification. Element screening can be done using real-time PCR or end-point PCR and may be multiplexed.

An alternative screening strategy would be to use (multiplex) event specific approaches. These can be strictly qualitative or (semi-)quantitative. Published examples include both qPCR and dPCR approaches (e.g. Querci *et al.*, 2009; Kluga *et al.*, 2011; Gerdes *et al.*, 2012; Kim *et al.*, 2010; Košir *et al.*, 2017a). The use, including both descriptions and recommendations, is discussed in further detail in annex A. 1.

The use of qPCR for cost-efficient GMO detection is challenged by the large and increasing number of GMOs on the market (including both authorized and un-authorized events). This is a problem because firstly each quantification with qPCR requires a standard curve of high quality. Secondly, due to the fact that in case of the detection and quantification of several GM-events <u>per species</u> (e.g. soybean) all these quantitative analytical results (per GM-event) sometimes need to be added (e.g. in order to evaluate correct food or feed labelling) and as a result the overall RSD (relative standard deviation) is the combination of all RSDs (as root of summed squares). This can be a problem because quantification is imprecise and difficult at near LOQ concentrations. Thirdly, PCR inhibitors co-extracted with sample DNA can cause a problem because the inhibitors can result in other amplification efficiencies than those observed for the standard curves, thus affecting the reliability of quantitative data. Fortunately, dPCR offers solutions to all these three major challenges.

Application of dPCR in GMO detection

With minor modifications, any probe-based qPCR assay can be converted into a dPCR assay. Many species-, element- and event-specific qPCR assays have been validated in a collaborative trial and are in current use in GMO-laboratories around the world. The EURL GMFF is maintaining a database of these qPCR assays (GMOMETHODS: EU Database of Reference Methods for GMO Analysis) and the ENGL members have a long history and experience with their use and know their strengths and weaknesses.

Notably, in contrast to qPCR, no standard curve is needed for dPCR in GMO quantification. This is because absolute DNA copy quantification performed with dPCR is inherently quantitative. When applied for one (or multiple) transgene(s) as well as for the reference gene (species specific), the GMO quantity relative to the reference gene can be calculated. Furthermore, partitioning increases the ratio of low concentration targets relative to non-target DNA. As a consequence, competitive exclusion of low concentration targets by high concentration targets is thus generally not a problem

in dPCR. PCR inhibitors affect the amplification efficiency, but only exceptionally lead to complete inhibition of the amplification reaction. For qPCR, however, partial inhibition would reduce amplification efficiency and consequently delay the accumulation of fluorescent signal, leading to underestimation of the target concentration. For dPCR partial inhibition is not a problem as long as the presence of a template copy in a partition yields a positive amplification signal detectable at the end-point.

Simplex dPCR for GMO detection

Most official quantitative detection methods published by the EURL GMFF are so far based on qPCR with hydrolysis probes (European Reference Laboratory for GM Food & Feed Status of dossiers; http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx). Several laboratories have tested the potential of dPCR in a chamber-based (cdPCR) or in droplet-based (ddPCR) format for the analysis of GMO. The ratio of absolute copy numbers of transgene per reference gene determined by cdPCR was found to be identical to the ratio measured by real-time quantitative PCR (qPCR) using a plasmid DNA calibrator. These results indicate that both methods could be applied to determine the copy number ratio of MON810 maize (Corbisier *et al*., 2010). Well characterised certified reference material bearing one copy of the transgenic target and one copy of the species reference target have been used to verify several assumptions of dPCR, some of which relate to Poisson statistics. The assumptions are (i) a large number of PCR reactions are analysed, as with most statistics a larger number of replicates/partitions means more power to discriminate small differences (Pagano and Gauvreau, 2000), (ii) random distribution of target DNA (Pinheiro *et al.*, 2012), (iii) independent segregation of target DNA (Bhat *et al.*, 2009), (iv) that every partition containing one or more DNA copies gives a signal and (v) that every molecule is double stranded DNA (Bhat *et al.* 2010). The absolute LOD and LOQ of cdPCR for GM quantification has been reported (Burns *et al*., 2010) and the applicability of ddPCR for routine analysis in food and feed samples has been demonstrated with the quantification of GMO (Morisset *et al.*, 2013). Finally, the contribution of several assay parameters (singleplex/duplex ddPCR, assay volume, thermal cycler, probe manufacturer, oligonucleotide concentration, annealing/elongation temperature and a droplet separation evaluation) have been evaluated for the quantification of GMO by ddPCR (Gerdes *et al.*, 2016).
The optimisation of DNA concentration in GMO quantification by dPCR around GM contents of 0.1 % is recommended. Detailed explanations are provided in annex A. 2 to generally illustrate such "lambda optimised approach".

Multiplexing of dPCR in GMO detection

While absolute quantification is often used (and needed) in other fields, GMOs are usually quantified relative to a species. When GMOs are quantified, dPCR offers an approach that qPCR cannot provide. For instance a duplex dPCR, where the species and the GMO of that same species are separately detected, e.g. with two separate fluorescence, will potentially yield the relative GMO concentration directly.

Many dPCR instruments currently available have two different fluorescent channels. Multiplexing therefore is limited compared to qPCR devices with multiple (e.g. five) channels. Duplex dPCR methods are conceptually similar to duplex qPCR methods and several methods to quantify one GM-specific target and one reference gene have been published (Morriset *et al.*, 2013; Gerdes *et al.*, 2016; Dalmira *et al.*, 2016). A triplex assay can be achieved on a two channel (e.g. FAM/VIC)

instrument by labelling one probe with FAM, a second probe with VIC and for the third target a 1:1 mixture of FAM and VIC labelled probes (Pretto *et al.*, 2015; Dobnik *et al.*, 2016; Whale *et al.*, 2016a). By analysing single labelled and double labelled partitions the three targets can be distinguished in a probe-mixing assay (Whale *et al.*, 2016a; also refer to 4.3.6).

By using different concentrations of probes (i.e. 100 and 300 nM) two targets can be distinguished based on absolute endpoint fluorescence read in the same channel (Dobnik *et al.*, 2016), (4.3.6, Figure 5). Four levels of fluorescence will be expected, negative, single positive for each target and double positives. This strategy can be extended to analyse four targets in two channels using high and low probe concentrations in each channel. This approach was applied using a method covering seven maize events (MON863, MON810, DP98140, MIR604, GA21, MON89034 and MIR162) and a maize reference gene (*hmgA*) in two assays. In a 2-D plot of fluorescence for two fluorescence channels, a total of 16 clusters of partitions can be expected (4.3.6, Figure 6). (Note: For the sake of quality assurance, this kind of analysis should be computerized and not performed on spreadsheets.) Dobnik *et al.* (2016) developed a computer-script to do automated threshold setting and quantification based on the various clusters.

The threshold for labelling of GM products in the EU (i.e. 0.9 % according to Regulation (EC) No 1829/2003) refers to the concentration(s) of EU authorized GMO(s) per ingredient (species). Although not (yet) common in EU enforcement laboratories, this theoretically opens the possibility to measure EU-authorized events of each single species together in one fluorescence channel and a species-specific reference gene in another fluorescence channel. This approach of multiplexing of dPCR in GMO analysis has successfully been demonstrated for EU-authorized GM maize (Dobnik *et al.*, 2015) and GM soybean (Košir *et al.*, 2017a). The latter study also included all soybean events falling under Regulation (EU) No. 619/2011 (at the time of publishing). Such methods will report the sum of a set of events (e.g. all EU authorised events) from one species (without taking stacked events into account). Alternative applications are also possible, e.g. by using dPCR multiplex for screening purposes and by subsequently re-analysing (quantitatively) only such samples, estimated to contain > 0.9 % GM material by singleplex or duplex dPCR (or qPCR) for the events detected.
The main advantage of duplex and multiplex dPCR assays are cost efficiency, due to the fact that multiple standard curves are not needed. In addition, for assays where the GM target(s) and the reference gene are analysed in the same partition (droplet or chamber), possible pipetting errors are reduced when relative concentrations are calculated.
Additional notes regarding *practical aspects of multiplex dPCR* are given in annex A. 3.

4.2.2 Digital PCR applications for monitoring human disease states

Digital PCR is ideally suited for identification of minor amounts of DNA targets such as point mutations, chromosomal translocations, DNA methylation and alternatively spliced mRNA. This has found applications in areas such as cancer diagnostics, non-invasive prenatal diagnostics, and assessment of microbial resistance. Additionally, dPCR can be used for the quantification of viral load (Trypsteen *et al.*, 2016; Sedlak and Jerome, 2013).

Digital PCR can also be used to measure and monitor copy number variations (CNV) which arise when an individual has one or more deletions/duplications of a particular genomic region (Usher and McCaroll, 2015) . CNV can occur naturally, but in humans, it can be used as an indication of disease state, for example as in aneuploidy (e.g. Down's syndrome) and cancer diagnosis (e.g. HER2 tumour

cell amplification in breast cancer). Current methods for measuring CNV include karyotyping, use of arrays, immunohistochemistry, PCR and Next Generation Sequencing (NGS). However, dPCR may provide a more rapid method for identifying CNV compared to traditional methods. Digital PCR can measure more accurately smaller fold changes than traditional qPCR, and therefore can be used for prenatal screening and tumour screening applications, as well as detection of CNV in cell-free DNA. Digital PCR is helpful for prenatal diagnostics without the need for invasive approaches, for example by sampling cell-free foetal DNA from the maternal blood plasma (non-invasive prenatal testing, NIPT) (Lo *et al.*, 2007; Zimmermann *et al.*, 2008; Fan *et al.*, 2009).

4.2.3 Digital PCR applications in the field of food virology

Hepatitis A virus (HAV) and norovirus (NoV) are important agents of food-borne human viral illnesses. No routine methods exist to culture these viruses from food matrices. Detection is therefore reliant on molecular methods using the reverse transcription-polymerase chain reaction (RT-PCR) followed by qPCR. Coudray-Meunier *et al.* (2015) published a comparative study of dPCR and qPCR for quantification of Hepatitis A virus and Norovirus in lettuce and water samples. For certain food matrices, e.g. bivalve shellfish, quantitative analysis is considered necessary for risk analysis. Quantification of levels of virus RNA by conventional real-time RT-PCR has been described in ISO 15216-1:2017. Due to the complexity of the method, it is necessary to include a comprehensive suite of controls, which need to be quantified as well. In the ISO standard mentioned, dPCR can be used as an option to quantify the nucleic acid control material.

The virus load of any sample can be estimated using dPCR. Preliminary data have shown that RT-PCR inhibitors do not have the same severe impact on quantitative data in dPCR compared to conventional real-time RT-PCR (Rački *et al*., 2014b). In principle, dPCR should allow quantification of any virus. Determination of virus loads is a prerequisite to estimate the risk connected to a certain food item and to verify the effectiveness of preventive measures, either at technological or at epidemiological level. A possible field of application besides HAV and NoV could be the detection of Hepatitis-E-virus in liver sausages, which is closely linked to food safety.

4.2.4 Digital PCR in the agricultural and environmental field

The fields of agricultural and environmental testing may be considered distinct but also share several similarities and to some degree overlap. They typically include a broad range of target organisms and complex matrices and the presence of inhibitory substances can be a great challenge (Strand *et al.*, 2011; Rački *et al.*, 2014b). The quantification of plant and animal pathogens is gaining importance, as regulatory bodies are shifting toward quantitative microbial risk assessments instead of mere qualitative detection.

Real-time PCR is used for water monitoring and microbial source identification with faecal indicator bacteria, plant, animal and human pathogens (Boben *et al.*, 2007; Strand *et al.*, 2011 and 2014; Huang *et al.*, 2016; Masago *et al.*, 2016; Wang *et al.*, 2016) and invasive species (Doi *et al.*, 2015). However, these assays are subject to bias introduced by reliance on quantitative standards, difficulty in multiplexing and inhibition. Digital PCR has shown to reduce all these problems and has proven to be appropriate in monitoring, faecal source identification and detection of pathogenic and invasive species in water samples (Morisset *et al.*, 2013; Rački *et al.*, 2014a; Doi *et al.*, 2015; Cao *et al.*, 2015).

Plant material, soil, and wastewater are matrices known to have high levels of inhibitors that reduce the likelihood of detecting and quantifying targets of interest (e.g. plant pathogens). Analyses with

dPCR have a higher resilience to inhibitors commonly found in such samples, in contrast to qPCR (Rački *et al.*, 2014b).

4.2.5 Digital PCR applications in the field of species identification

Identification of species, including plants, animals or microorganisms, is important in order to be able to monitor food integrity and to detect food fraud. However, monitoring the compliance of the relevant EU labelling legislation by demonstrating the presence or absence of a particular species or products derived thereof, is also highly important. Species identification is important to define the origin and traceability of raw materials and derived food products as well as to check for the potential unintentional occurrence of other species (contamination). The most extensive use of molecular techniques (genomic and proteomic techniques) is for determination of species and botanical origin, while all other techniques are mostly dealing with adulteration and geographical origin (Danezis *et al.*, 2016). In some cases, such as food allergens, it is also a food safety issue (EU Regulation 1169/2011). Finally in the context of protection of endangered species, detection and identification of organisms is essential too (Staats *et al.*, 2016 and references therein).

The adventitious presence of traces of species in the food supply chain often cannot be avoided completely. For food allergens and endangered species, the presence of even minute quantities may be unacceptable. Quantification based on determination of species DNA copy numbers is an attractive approach to meet these needs. The application of dPCR in this evolving field will most likely facilitate implementation of cost-effective and reliable analyses in the near future (Scollo *et al.*, 2016). In the context of food allergens, one should distinguish between
a) checking compliance with the legislation on the labelling of ingredients, and
b) checking the accidental presence/contamination of foods with allergens.
The latter, precautionary allergen labelling principle (PAL), is voluntary and currently not required by any legal act in the EU (at the time of publication). Digital PCR might be a very interesting tool for checking for compliance of product composition with its corresponding ingredient list and for searching for other, potentially unintended components. Conversion factors are needed in order to be able to compare such results with results obtained by analytical methods detecting other target molecules. The use of dPCR to checking for accidental presence of food allergens should probably be implemented primarily as a screening tool that needs to be complemented with LC/MS methods.

4.2.6 Digital PCR applications in the field of microbiology

In Europe, thermophilic *Campylobacter* are one of the major bacterial food pathogens (EFSA & ECDC 2015). Hygienic measures in the whole food chain, starting at primary production, are considered as the most effective tool to fight campylobacteriosis. Risk assessment in slaughterhouses is based on *Campylobacter* quantification by conventional microbiology. This methodology, however, is prone to underestimation of the infectious bacterial contamination because “viable but non cultivable” *Campylobacter* are not detected. This problem also affects several other foodborne pathogens. Qualitative molecular methods are available and standardized (ISO/TS 13136:2012; ISO/TS 18867:2015). The application of dPCR may provide a step towards quantification in this important field.

Several food-borne bacterial pathogens such as *Bacillus cereus*, *Staphylococcus aureus*, and *Clostridium perfringens* owe their pathogenicity to the production of toxins after multiplication. Several toxigenic fungi such as *Aspergillus*, *Fusarium* and *Penicillium* spp. also affect food safety. As

the detection of toxins in food can be cumbersome in routine testing, an attractive alternative is the use of microbial counts as rough estimates for the food safety. The microbial counts, however, are not strictly related to toxin production. Not all strains are toxigenic, and the toxin production can also vary among strains and with environmental conditions. Some toxins produced by foodborne pathogens (bacteria and fungi) can be persistent to processing methods like heating, irradiation or high pressure that kill the microorganisms (e.g. trichothecene mycotoxins produced by *Fusarium* spp., enterotoxins produced by *Staphylococcus aureus*, and emetic toxin produced by *Bacillus cereus*; Stenfors Arnesen *et al.*, 2008; Pinchuk *et al.*, 2010; Hennekinne *et al.*, 2012; EMAN, 2015). Thus, the toxins may be present also in the absence of viable pathogens. DNA is also relatively persistent to such treatment. Thus, DNA based detection/quantification may be very useful to assess the potential presence of toxins. The advantages listed above for dPCR compared to qPCR suggest that dPCR may also prove superior to qPCR for the enumeration of foodborne pathogens.
In microbiology, dPCR has been applied – besides bacteria, viruses and fungi – to parasite analysis, e.g. for the sensitive detection and identification of parasites from human blood (Wilson *et al.*, 2015).

5. Technical aspects of digital PCR

This part of the document goes into more detail on each technical aspect of dPCR, giving background and guidelines on each facet of the GMO analytical chain. Extra attention is devoted to differences from the standard qPCR approach.

This part of the document is subdivided into six modules, each representing a step in the analytical process from sample preparation and analysis to results generation and their interpretation. The six sections are:

1. Theoretical basis of dPCR;
2. DNA extraction;
3. PCR;
4. Results and performance requirements;
5. Data interpretation and reporting;
6. MIQE guidelines for publishing dPCR data.

5.1 Theoretical basis of digital PCR

5.1.1 Statistics and assumptions

Central to the analysis of dPCR is the Poisson distribution, a discrete probability distribution that expresses the probability of a given number of events[1] occurring in a fixed interval of time or space. It assumes that these events occur with a (fixed) average rate and independently of time since the last event. The Poisson distribution is given by:

$$P(k) = \left(e^{-\lambda}\right) * \frac{\lambda^k}{k!} \qquad (1)$$

[1] *Event* is a statistical term and does not refer to GM event in this paragraph

Where $P(k)$ is the probability to observe exactly k targets (e.g. molecules), λ is the average number of events per interval and k is the actual number of events. In the context of dPCR, the event is "presence of the target sequence" and λ is the average number of targets per partition.
Analysis of dPCR results is based on the assumption that the distribution of the target sequence over the partitions is a nearly perfect Poisson process. Let n_{tot} be the total number of partitions for which we have a read-out and n_{neg} the number of negative partitions, we then estimate p_0 (probability of an empty partition) and λ (the average number of targets per partition) as:

$$p_0 = \frac{n_{neg}}{n_{tot}} \tag{2}$$

$$\lambda = -\ln p_0 \tag{3}$$

The ratio of GMO in the sample (in haploid genome equivalents) is then given by

$$Ratio = \frac{\lambda_{tr}}{\lambda_{en}} \tag{4}$$

Where λ_{tr} and λ_{en} are the concentration estimates for the transgene and endogene, respectively. Note that calculating the GM ratio does not require the droplet volume to be known, it is however assumed to be constant. This assumption, however, is not absolutely correct (Dong *et al.*, 2015). Variability in the droplet volume will increase the quantification uncertainty. Most manufacturers have taken precautions to keep this variability within strict limits (e.g. excessively large or small droplets may be removed from analysis). The 95 % confidence bounds (CB) can be calculated for each of the individual λ estimates:

$$\lambda_{en,\ CB} = \lambda_{en} \pm 1.96 \sqrt{\frac{n_{tot} - n_{neg}}{n_{tot} * n_{neg}}} \tag{5}$$

$$\lambda_{tr,\ CB} = \lambda_{tr} \pm 1.96 \sqrt{\frac{n_{tot} - n_{neg}}{n_{tot} * n_{tneg}}} \tag{6}$$

Whereas the CB for the ratio of transgene to endogene is obtained using Fieller´s theorem:

$$Ratio_{CB} = \frac{\lambda_{tr}\,\lambda_{en} \pm \sqrt{\lambda_{tr}^2\,\lambda_{en}^2 - \left(E^2 - \lambda_{en}^2\right)\left(T^2 - \lambda_{tr}^2\right)}}{\lambda_{tr}^2 - T^2} \tag{7}$$

Where

$$E = \left|\lambda_{en} - \lambda_{en,\ CB}\right| \quad (8) \quad \text{and} \quad T = \left|\lambda_{tr} - \lambda_{tr,\ CB}\right| \tag{9}$$

with CB being the relevant confidence bound (upper or lower). Where E is the absolute difference between the concentrates estimates of the endogenous target and the confidence bound associated to that estimation and T is the absolute difference between the concentrate estimates of the transgenic target and the confidence bound associated to that estimation.

As an alternative to Fieller's theorem, an *ad hoc* confidence interval can be used (ratio ± 1.96 ∗ standard deviation of the ratio). Such a confidence interval is more straightforward to calculate and has been shown to have 95 % coverage when a sufficient number or repeats is used to obtain the standard deviation of the ratio (e.g. four repeats: two subsamples analysed in duplicate; see Lievens *et al.*, 2016).

5.2 DNA extraction

Methodologies for investigating the quality of the extracted DNA to be analysed in qPCR have been extensively described. Related acceptance criteria have been elaborated with particular focus on applications for GMO detection (Marchesi *et al.*, 2015). The number of references about this topic in relation to dPCR is still limited, but some experimental evidence indicate that dPCR is less sensitive to PCR inhibition compared to qPCR (Nixon *et al.*, 2014; Rački *et al.*, 2014b; Iwobi *et al.*, 2016). As target concentration in dPCR is calculated from endpoint positive and negative reactions, one can assume that partial PCR inhibition will have less impact on quantification. Generally, the same DNA quality requirements should be applied to dPCR analysis as for qPCR analysis.

There are many methods for extraction of DNA that will yield high quality DNA, i.e. non-degraded and free of excess salts, proteins or polysaccharides (e.g. Murray and Thompson, 1980; Sambrook and Russel, 2001). In addition, DNA quality also depends on sample processing that affects structural integrity and physical-chemical purity of the extracted DNA.

5.2.1 Sample preparation

Concerning sample preparation there are no specific requirements for dPCR, one can therefore refer to the document developed within the ENGL: Guidelines for sample preparation procedures in GMO analysis (Berben *et al.*, 2014).

5.2.2 DNA yield

Regarding the quantity and concentration of extracted DNA, no special requirements are recommended for dPCR. The yield should be at least as much as is required for the subsequent PCR analyses (Marchesi *et al.*, 2015).

5.2.3 DNA structural integrity

With regard to structural integrity, considering that the amplicon length of dPCR systems is identical to their qPCR counterparts, the effects of DNA fragmentation are expected to be the same for all PCR systems.

Under certain circumstances, it may be necessary to perform a restriction digest of the DNA before partitioning in droplets or chambers to improve the accuracy of the measurement for the following reasons:

a) Decreasing the viscosity of the DNA solution;
Hindson *et al.* (2011) reported changes of droplet volume and possible decrease of accuracy of measurement when using more than 66 ng DNA (human genomic DNA) in a 20 µL ddPCR due to increased viscosity of the DNA solution. By restriction digest this viscosity could be reduced and even much higher DNA concentrations (1 µg) could be used in ddPCR without affecting the droplet

volume. BioRad recommends restriction digestion whenever DNA input is greater than 66 ng per 20 µL reaction (Kaihara *et al.*, 2016).

b) Separation of linked gene copies;
If the DNA molecules contain linked gene copies (e.g. tandem gene copies) one positive droplet or partition will contain multiple copies (e.g. two linked copies will be counted as one copy). This can be overcome by restriction digest which leads to a physical separation of such gene copies (BioRad Bulletin 6277 Rev A, 2012) and thus enables independent segregation.

c) Improving the accessibility of the DNA when working with supercoiled plasmids;
By linearisation of plasmid DNA by restriction digest the accessibility (and thus the efficiency of primer/probe binding to the DNA) can be improved, leading to more accurate quantification of the plasmid (Kaihara *et al.*, 2016);

If a restriction digest of DNA is performed prior to dPCR there are three considerations that should be taken into account in the choice of enzyme for a particular locus:

(1) The enzyme should not cut within the PCR amplicon sequence itself;

(2) It is best to use an enzyme that is insensitive to methylation to avoid incomplete fragmentation due to methylation of the target DNA; and

(3) In some instances, it is optimal to digest the target copy to the smallest size fragment that fully contains the amplicon footprint [sequence] – preferable under a few hundred base pairs (BioRad Bulletin 6277 Rev A, 2012).

Often restriction enzymes with 4-base and 6-base recognition sites are used.

However based on practical experience restriction digest of DNA is not mandatory and should be considered on a case-by-case decision (Jacchia *et al.*, 2018).

Note: Random shearing (fragmentation) of the DNA by sonication and a column-based method has been reported to improve the accuracy of measurement in analysing mitochondrial DNA (Vitomirov *et al.*, 2017).

5.2.4 Purity of DNA extracts

With regard to physical-chemical purity of the DNA extract, PCR (and in particular qPCR) is known to be susceptible to inhibition due to the possible presence of impurities in the extracted DNA (Huggett *et al.*, 2008; Kennedy and Oswald, 2011). Notwithstanding the still limited experience, dPCR is demonstrated to be less prone to inhibition (Nixon *et al.*, 2014; Rački *et al.*; 2014b; Iwobi *et al.*, 2016). Until there is sufficient evidence to conclude otherwise, acceptance criteria already set for qPCR are considered valid also for dPCR.

5.2.5 Additional recommendations

Some methods for DNA extraction might interfere with droplet generation. The impact of the extraction method on downstream workflow should therefore be evaluated once in the process of implementing dPCR in a laboratory. This can be done by extracting a) a certified reference material, and b) a food sample, with the standard DNA extraction methods applied in a specific laboratory.

Two DNA dilutions should be tested each for a) and b). The droplet plots between DNA extraction methods should be compared in terms of signal (positive and negative droplets) separation and signal intensity. If an extraction method shows significantly deviating (poor) performance compared to others, then this method should not be used for extraction of DNA for dPCR.

Another important consideration is whether the DNA subjected to analysis is double or (partially) single stranded when partitioned. The different strands of fully denatured molecules may be divided over different partitions, leading to two positives for a single (double stranded) target copy. As a consequence, if a significant portion of the sample DNA is denatured, the amount of target will be overestimated if the user assumes that only double-stranded DNA is quantified. This effect can occur, when DNA is exposed to sustained elevated temperatures or alkaline solution which therefore should be considered and avoided when designing dPCR experiments for absolute quantification of DNA copy numbers (Bhat *et al.*, 2011).

The options are to either validate the method that produces 100 % of single stranded DNA and then apply a 2-fold factor to concentration calculations or to validate a method that produces 100 % of double stranded DNA (Holden *et al.*, 2009)

Another important assumption is that the DNA target molecules segregate independently. If the DNA molecules are concatemers or physically bound to each other, one positive droplet or partition would contain multiple copies (e.g. two linked copies will be counted as one copy).

5.3 PCR

The set-up of a dPCR follows very closely the assembly of a qPCR. The reaction ingredients include: template nucleic acid, forward and reverse primers, a hydrolysis probe (e.g. TaqMan, not included for detection strategies using intercalating dye), sterile nuclease-free water to adjust the reaction volume, and the master mix. The latter includes the reaction buffer, bivalent cations, nucleotides, and a DNA polymerase (plus the DNA intercalating dye – e.g. SYBR® Green or EvaGreen – if this detection strategy is selected). In addition, technical additives may be included, depending on the master mix and the digital PCR format.
The main difference from qPCR is the step of partitioning the reaction mix into a large number of chambers or droplets which typically occurs shortly before the amplification itself.

5.3.1 Master mix

In cdPCR, a loading reagent is added to the reaction mix to facilitate the microfluidics of the partitioning. In droplet-based systems, specific surfactants are often included in the master mix in order to add stability to the droplets and to minimize coalescence once they are formed.
As a consequence, proprietary master mixes associated with specific platforms are commonly used for dPCR and the use of other non-proprietary master mixes is not recommended by the instrument manufacturers. However, a few instrument platforms (e.g. Constellation, Naica, and Raindance systems) are not bound to the use of dedicated master mixes. At least for Constellation, the master mix used should contain ROX dye.
The JRC method database (GMOMETHODS) contains protocol information for more than a hundred validated GMO assays. The large majority of these make use of some type of commercial master mix optimized for qPCR. Protocols already validated for qPCR may need to be amended when used with another master mix.

Commercial master mixes may include non-declared components that can yield false positives. For example, the presence of bovine serum albumin (BSA) may yield positive signals for a dPCR targeting bovine. Special attention should therefore always be given to unexpected results for negative controls when modifying a qPCR protocol for dPCR application.
In the case of GMO methods, this can be done by evaluating the specificity of the detection method for the taxon and for the GM target, taking into account the following aspects:

Composition of the master mix. While the exact composition of master mixes is generally not known, the concentrations of the key components is usually stated ($MgCl_2$, dNTPs, polymerase, buffer). If their concentrations are comparable (within 10 % variation) with the conditions of the validated conditions (master mix), then the reassessment of specificity is not required. Otherwise, an experimental check on selected targets is recommended (Onori *et al.*, *in preparation*). It is advisable to select and check those targets, which have the most sequence similarities (primer and probe sequences) compared to the method to be implemented. This can be done by bioinformatic analyses using e.g. the molecular database CCSIS (Central Core DNA Sequence Information System) of the EURL GMFF or one of the NCBI databases (National Center for Biotechnology Information).

5.3.2 Primers and probe concentrations

For the design and selection of primers and probes, one should use the same rules as for qPCR assays (in terms of target-matching, base composition, length, melting temperature, absence of secondary structures and self- and inter-complementarity and specificity (Degen *et al.*, 2006)). However, primers and probe concentrations in dPCR are usually higher than in qPCR. Higher primer and probe concentrations increase the intensity of the end-point fluorescence signal and thus allow better separation of the background noise from specific signals, aiding more accurate quantification of the target. Tests carried out in ddPCR (BioRad QX200) on twelve assays validated by the EURL GMFF in qPCR have shown that the best results were obtained when the final concentration of primers were set around 500 nM per reaction while keeping the primer:probe ratio the same as the validated qPCR conditions (Lievens *et al.*, 2016).

Manufacturers may recommend certain combinations of reporter and quencher. For instance, in the Bio-Rad QX200 ddPCR system, it is recommended to NOT use TAMRA as a quencher as its emission overlaps with the emission of HEX (or VIC®), resulting in additional background signals in the second fluorescence channel. This deteriorates cluster separation and peak resolution. Instead, the use of non-fluorescent quenchers is recommended. Nevertheless, this relates not only to dPCR but can be considered as a general recommendation as TAMRA contributes to an overall increase in fluorescence background. Changing the fluorescent dye or quencher during the transfer from qPCR to dPCR should generally not have any effect on the performance, thus additional validation is not needed, unless the change affects the annealing temperature of the probe (e.g. MGB probes). In such case additional optimisation and validation are necessary.

For the transfer of existing qPCR methods into a dPCR format, it is recommended to initially use the concentrations from the validated qPCR method, and to run a primer/probe concentration gradient if it is deemed necessary to improve the end-point fluorescence values of the positive partitions.

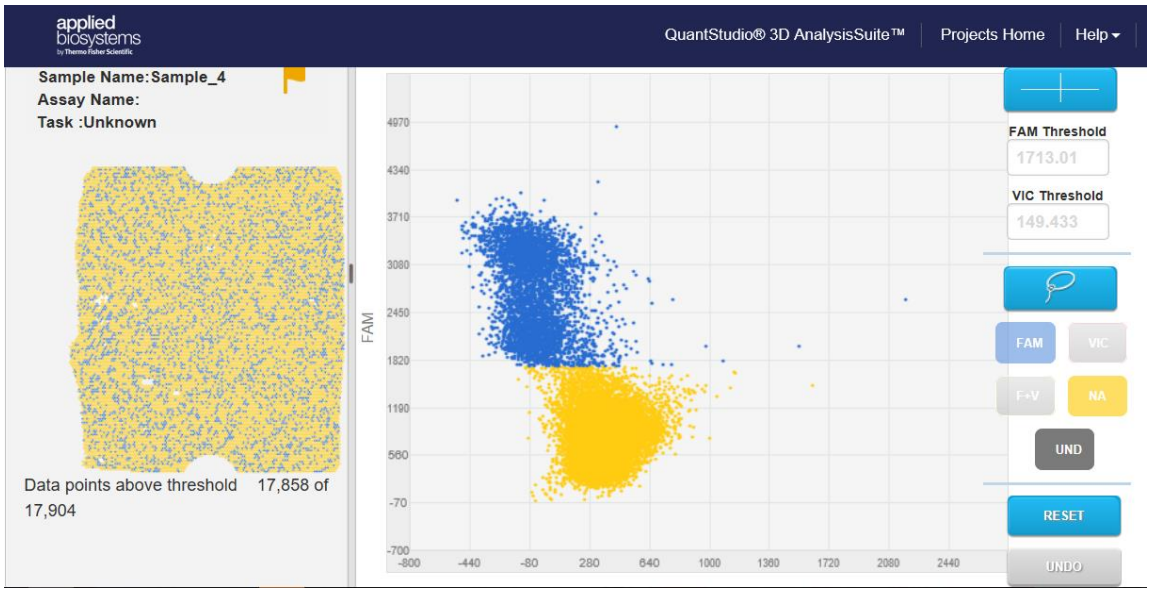
Primer and/or probe concentrations. If primer/probe concentrations are (substantially) changed, additional performance parameters should be experimentally assessed (e.g. specificity and trueness, Hougs *et al.*, 2017).

Primer and/or probe sequence. In case of changing of primer/probe sequence, a full validation of such a new method is needed. For method performance parameters, see e.g. Marchesi *et al.*, 2015

5.3.3 Temperature cycling program

In general, the temperature profile used in qPCR can be directly applied to dPCR. However, one should always check the manufacturer's recommendations for specific requirements. For example, in droplet-based systems, a final step at higher temperature (e.g. 98 °C, 10 min for the Bio-Rad QX100/200 ddPCR systems) may be mandatory to further stabilize the droplets prior to reading them. There may also be recommendations concerning the ramping rate to ensure uniform heating of the partitions (droplet or chamber).
It is also advisable to run a minimum of 45 amplification cycles, in order to have sufficient separation between positive signals and background noise. An example is illustrated in Figure 2.

a)



b)

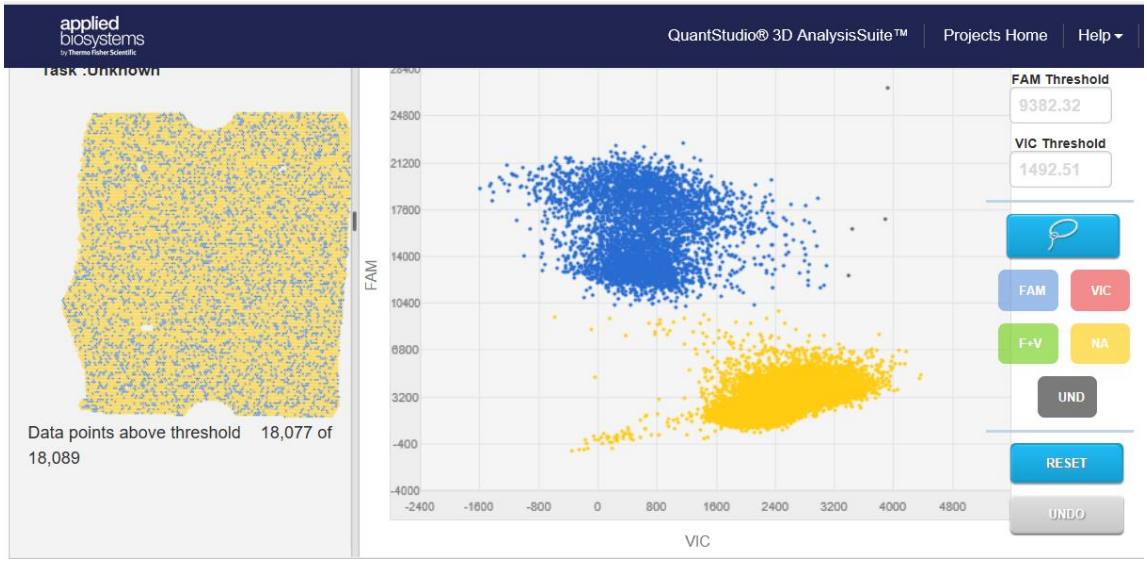


Figure 2: Signal separation in cdPCR for MON 810 (Primers 300 nM, Probe 180 nM) performed with QuantStudio™ 3D Digital PCR System either run with 39 (a) or 45 (b) PCR cycles.

Annealing temperature. The annealing temperature contributes to the specificity of PCR reactions, and it should ideally be maintained in line with that of validated methods. However, in the case of co-amplification of a secondary target (multiple clouds of positive droplets) one can increase the annealing temperature in order to have sufficient separation between positive signals and background noise (Figure 3). If the deviation in temperature falls within the robustness range [assessed for the validated method], there is no (general) need for specificity testing. Nevertheless the specificity should be tested with a method whose annealing temperature is decreased. If the annealing temperature of a method is increased, it is not necessary to test the specificity because the new conditions are more stringent for primer annealing (Onori *et al.*; in preparation).
Possible effects of different annealing temperatures on signal (positives) to background (negatives) separation is illustrated in Example 2, Figure 4.

Example 1: *Acp*1 co-amplification of a secondary target

Figure 3 below shows an annealing temperature gradient experiment with the same amplification system *acp1* – acyl carrier protein, specific to cotton. This gene is present in two almost identical copies in cotton. One target has a perfect match to the designed PCR method while the other one contains four SNPs. The dPCR was run at temperatures ranging from 62 °C to 56 °C. While at the highest annealing temperature, the *acp1* cloud is visible (blue dots, where each dot represents the fluorescent signal of one droplet) in addition to the background fluorescence (grey dots), at decreasing annealing temperatures a second cluster of positive droplets (grey dots above the Ch1 amplitude of 3,000) stems from the co-amplification of the closely related gene at lower efficiency. As the annealing temperature lowers, its fluorescence gradually increases and gets closer to the *acp1* specific cluster of droplets. The highly stringent annealing temperature conditions at which the reaction was run at 62 °C impeded the amplification of the secondary target.

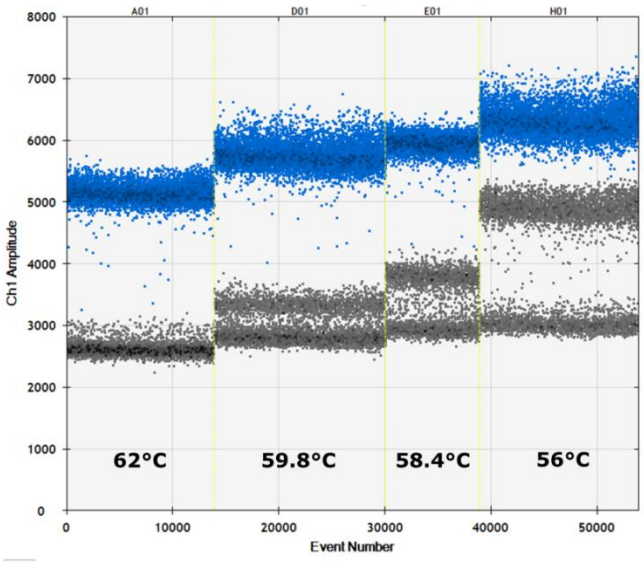
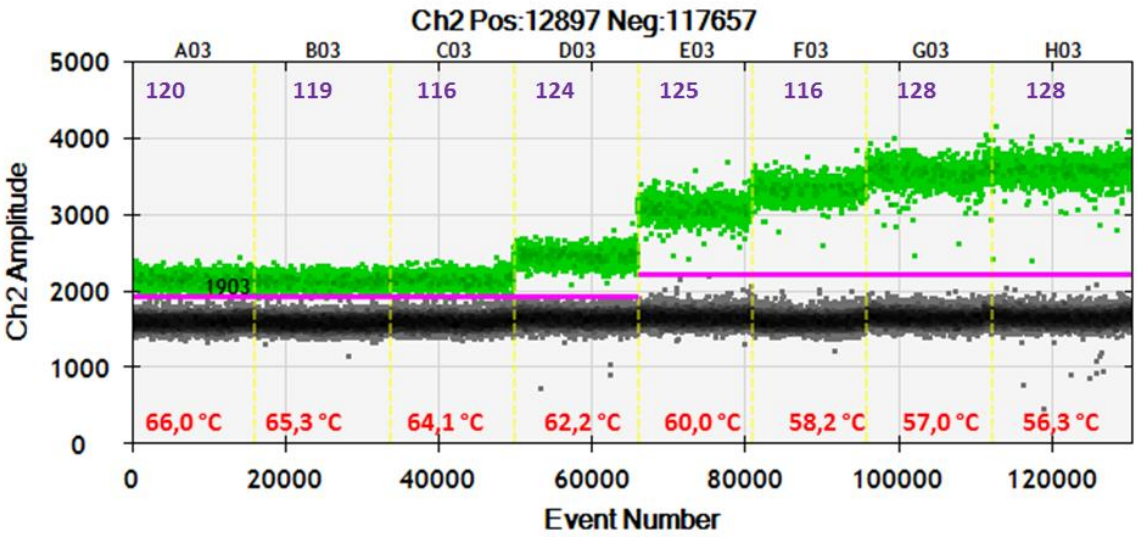


Figure 3. Annealing temperature gradient of the cotton-specific *acp1* amplification system in ddPCR. As the annealing temperature is raised the specificity of the reaction increases and efficiency of the co-amplification is reduced, until the undesired droplet population merges with the negative population.

Example 2: Temperature dependent signal separation of a *lectin* and MON 87769 soy-specific duplex ddPCR

Figure 4 below shows an annealing temperature gradient experiment – using a 100 % MON 87769 soy certified reference material – with a *lectin* and MON 87769 soy-specific duplex ddPCR, run at temperatures ranging from 66 °C to 56.3 °C. With decreasing annealing temperature, signal separation (positives and negatives for both *lectin* and MON 87769 reaction) increases to finally reach a maximum separation plateau. This increased separation reflects a more efficient primer and probe binding to the target DNA. Even at temperatures significantly below the validated 60 °C annealing temperature, no unspecific signal populations are observed.

a) *Lectin* specific reaction:



b) MON 87769 specific reaction:

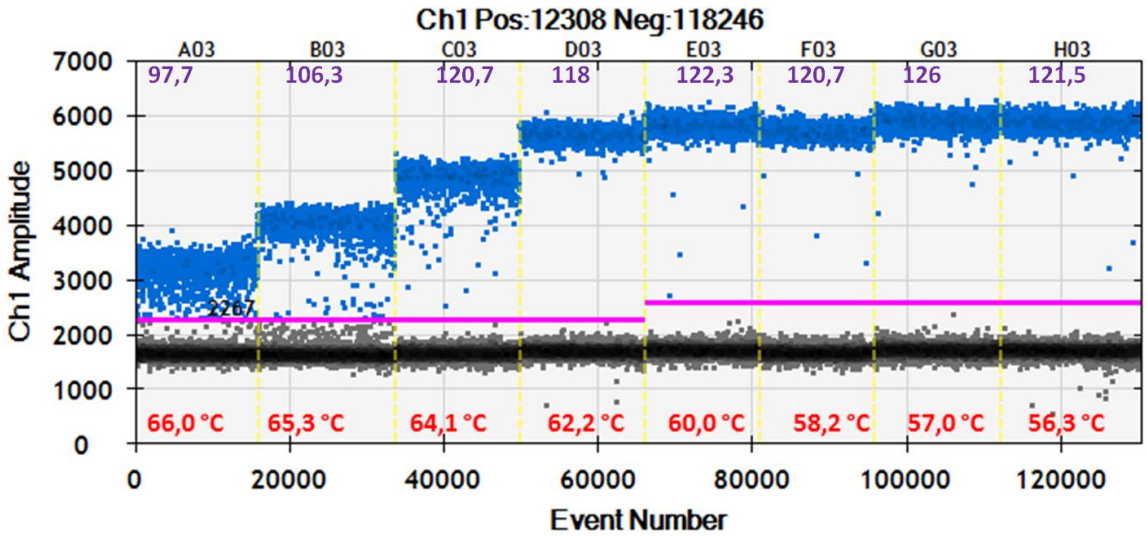


Figure 4. Annealing temperature gradient of the *lectin* (a) and MON 87769 (b) soy-specific amplification system run in duplex ddPCR with a 100 % MON 87769 soy certified reference material. Purple numbers reflect calculated DNA copy numbers per microliter PCR reaction. Purple lines represent the threshold set for separating positive from negative droplet populations.

5.3.4 The use of control samples

Although quantification with dPCR is not dependent upon the generation of a calibration curve for results interpretation, it still requires the use of appropriate positive controls to provide evidence that the experiment has worked correctly. In addition, negative controls should always be used in a dPCR experiment to test for false positives. The use of control samples is essential for dPCR to control the appearance of the above-mentioned effects. In the case of routine GMO quantification, as for the qPCR, the controls described in ISO 24276 should also be used in dPCR. When an assay is being transferred from qPCR to dPCR, additional control samples should be used (e.g. certified reference material or samples with known GM content), enabling a direct evaluation of dPCR performance.

5.3.5 Plasticware

It appears that the brand of the plasticware and especially that of the tips can be of importance to reach good results. Due to the nature of the instruments, reactions should be set up in plasticware appropriate for use with digital PCR instruments according to the manufacturer´s recommendations.

5.3.6 Considerations for multiplexing

Multiplexing allows for simultaneous identification and quantification of more than one target in a single reaction. The majority of dPCR systems currently on the market can detect fluorescence in two colour channels, FAM and HEX (VIC®), and some have an option of detecting a third fluorophore. Duplex reactions can easily be implemented; fluorophore-specific signal is acquired through either channel, computed by the software algorithm and graphically represented in a two-axis plot of FAM and HEX amplitude.

Although the availability of only two-colour channels (FAM and HEX) limits the choice of the reporters for TaqMan probes, more complex levels of multiplexing can be attained in dPCR by

labelling two or more probes, belonging to different target-specific assays, with the same reporter and varying the concentration of the probes (see Figures 5 and 6 for examples; also see Whale *et al.*, 2016a). This results in a spatial separation of the respective clusters of amplified targets on the basis of their florescence level. Identification of the clusters with their specific assays is established in simplex reactions during the optimisation stage of the test where the positioning of each cluster with the adjusted probe concentration can be easily identified in the two-dimensional plot. It is possible to successfully combine dPCR assays into multiplex assays if different reporter fluorophores (e.g. FAM and HEX) and probe concentrations are used for different targets. This, however, requires careful optimisation, and the complexity increases exponentially with the number of included targets. Separating the different targets into clearly identifiable clusters is necessary for reliable identification and quantification. Such multiplexing is theoretically possible with any dPCR format, but has so far only been demonstrated with ddPCR (time of publication).

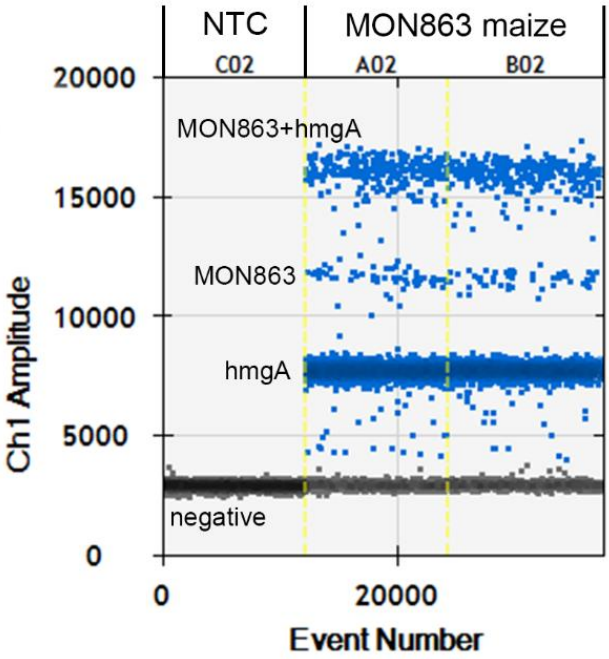


Figure 5: Example of duplex droplet readout in one fluorescence channel for three wells (one well for sample NTC and two wells for sample MON863 maize). Both probes (for *hmgA* and MON863) were labelled with the same fluorophore (FAM), but in the reaction mixture for *hmgA* the concentration was 100 nM for the probe and 300 nM for the primers (low), whereas for MON863 the concentration was 300 nM for the probe and 900 nM for the primers (high). In the 1D amplitude view, the positive droplets for the low primer/probe concentration target (*hmgA*) appear as cluster just above the negative one. The next cluster (above the *hmgA* cluster) are droplets positive for the high primer/probe concentration target MON863, whereas the topmost cluster are droplets positive for both of the targets. To achieve the best possible separation optimization of primer and probe concentrations is needed (Dobnik *et al.*, 2016).

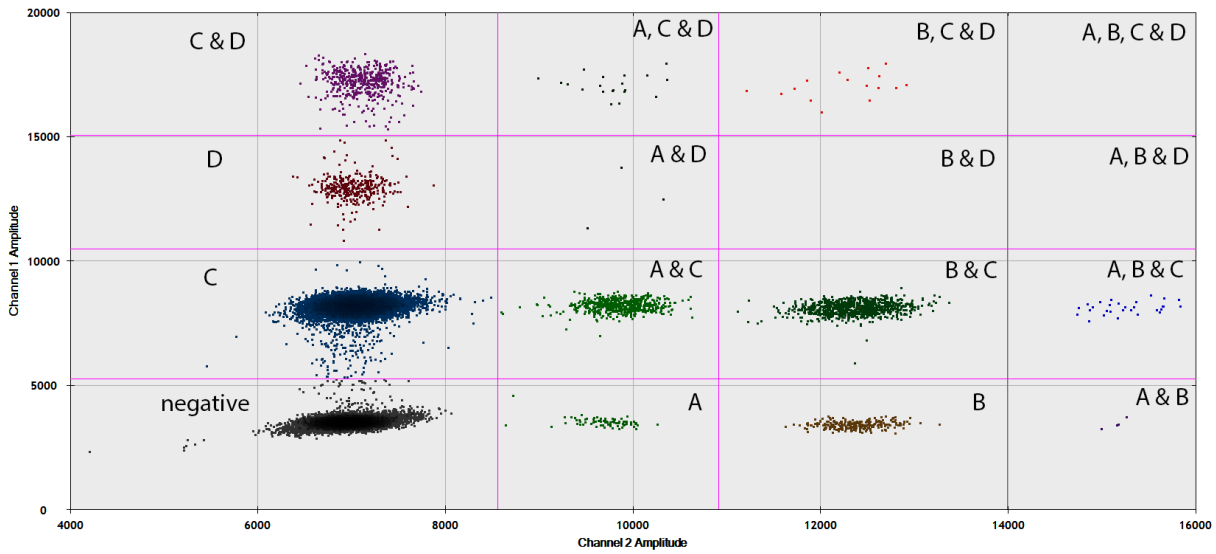


Figure 6: Graphical representation of possible target combinations in droplet clusters and their area of appearance in the 2-D amplitude view. When primer and probe concentrations have been optimized for the best separation of clusters in individual channels, the assays may be combined to achieve the highest level of multiplexing (4-plex). In case of four targets (A, B, C and D), theoretically there are sixteen different possible clusters where the different combinations of targets might appear after readout. Presence or absence of the clusters depends on the ratio of targets in the sample and their initial concentration (Dobnik *et al.*, 2016).

The Regulation (EC) No 1829/2003 specifies that quantification of GM material shall be based on the concentration per ingredient (not per event). In practise, the ingredient is interpreted as species. Consequently, for the EU-authorised GMOs testing for legal compliance can be done – although not common practice in EU enforcement laboratories – using duplex (multiplex) quantification where the species specific reference gene is quantified in one channel and the authorised GMOs belonging to that same species are quantified simultaneously in the other channel, as exemplified for maize (Dobnik *et al.*, 2015) and soybean (Košir *et al.*, 2017a).

5.4 Results and performance requirements

Digital PCR is still only a PCR in a different format; thus all of the pre-written minimum performance requirements (MPRs) (Marchesi *et al.*, 2015) also apply in this field, with the exception of amplification efficiency that is not as important because dPCR is an end-point reaction. Digital PCR assays must therefore be compliant – where appropriate – with these MPRs in order to be suitable as a method for quantification of GMOs. They should be valid not only for GMO detection methods, but for all applications of dPCR as they are technical criteria in order to ensure that the method works properly before proceeding towards determination of parameters such as LOD.

Nevertheless, dPCR has some of its own specific issues which require setting some additional performance requirements, which must be fulfilled before proceeding to validation. If the PCR assay is specific and efficient, the main source of error in digital quantification is caused by the misclassification of partitions. To allow accurate classification of partitions and thus reliable quantification the method should meet acceptance levels within several criteria associated with the following simplex dPCR results: (I) single amplification product (there should only be two fluorescence populations), (II) peak resolution (as a measure of the separation between positives and negatives), and (III) the amount of stragglers or 'rain' (i.e. droplets that have an intermediate fluorescence and do not seem to belong to either the positive or negative population). Two further

criteria that apply to each reaction (post run evaluation) are: (IV) target concentration and (V) fraction of sample compartmentalized. In the following sections, we will define these specific criteria and set their limits, most of which are based on the qPCR MPR stating that the relative error should be below or equal to 25 %.

In the course of method validation for the measurement of the dPCR-specific performance (e.g. resolution, amount of rain), it is recommended to select a target concentration, which would give the λ value around 0.7. This corresponds to a situation in which approximately half of the partitions are positive and thus allows gauging both the rain and the dispersion of the droplets without being biased by the fact that one population is larger than the other.

Classification of partitions as positive or negative is based on the threshold, which should be set just above the cluster of negative partitions. NTC sample, with only negative partitions, can help in setting the threshold, however, an inspection of all wells/panels is recommended. Fluorescence amplitude of individual wells/panels might be slightly higher or lower than NTCs, thus in such cases the threshold can be adjusted individually to avoid misclassification of some droplets.

A reaction is considered positive, when the number of positive partitions exceeds 2. There is no clear consensus on this value, however, this proposed value was determined based on experiments with a statistically significant replication level negative samples (0 % GM Material and NTCs, Dobnik *et al.*, 2015) and corresponds to the theoretical limit of detection of PCR.

5.4.1 Amplification product of a single target

In a qPCR reaction, the fluorescence measured is the sum of all amplification processes in the reaction mixture. It is therefore impossible to distinguish between different amplification products arising from the same primer probe combinations in the same reaction (e.g. the amplification of closely related sequences). In dPCR, the compartmentalisation allows such a distinction. Unintended amplification products (i.e. non-perfect match) usually amplify at a lower efficiency. This results in an endpoint fluorescence that is lower than for the actual target (after a standard run of 45 cycles). As a consequence, these amplifications show up in dPCR as an additional (distinct) population of droplets with fluorescence values between negatives and the true positive (see Figure 7 and Example 1, Figure 3). The presence of multiple populations of droplets may complicate the digital analysis, affect the separation of positives from negatives, and ultimately lead to misclassification of droplets. Therefore, dPCR methods for GMO quantification should only amplify a single target unless the aim is to perform multiplexing.

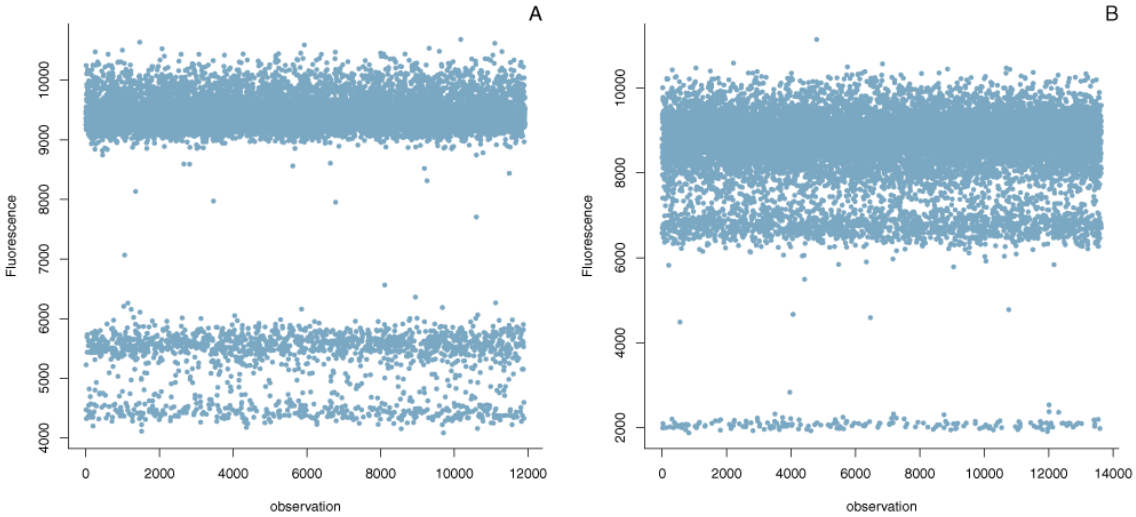


Figure 7: Illustration of multiple fluorescence populations. **Panel A** shows the results for the *acp1* target, an additional population of fluorescence measurements is visible with values closely situated to the negatives. **Panel B** shows the results for the cruciferin (*cruA*) target, an additional population of fluorescence measurements is visible with values closely situated to the positives. Contrary to "rain", this kind of intermediate fluorescence is not uniformly spread between negatives and true positives, but appears in a more discrete manner.

5.4.2 Amount of rain

In many dPCR reactions there are partitions that seemingly fail to belong to either the positive or negative population. These partitions have an intermediate fluorescence level and are colloquially referred to as "rain" or "drag" (e.g. see Figures 3 and 4). Rain represents droplets with intermediate fluorescence that defy straightforward classification as either positive or negative. In contrast to unintended amplification products, rain does not seem to have a pronounced distribution but it is often equally spread between positives and negatives. The occurrence of rain may for example be attributed to a late PCR onset due to partial inhibition in a certain amount of droplets (Dreo *et al.*, 2014 and Dingle *et al.*, 2013). In addition, experiments indicate that after a standard reaction (40 or 45 cycles) the cloud of negatives may still house a population of rain partitions that have not yet had the time to accumulate fluorescence above the baseline level. Thus, the main question relating to how much rain we can tolerate becomes: how many rain partitions are "hiding" in the negative cloud (and are thus always misclassified).

In order to set a limit to the amount of rain that can be allowed in dPCR reactions, we consider the misclassification of partitions. Simulation experiments show that, at the 1 % quantification level, approximately 2.5 % rain (percentage of total droplets) can be tolerated before 25 % quantification bias is reached (Lievens *et al.*, 2016). Hence, we propose the latter as a rule of thumb for the maximum amount of rain in a given reaction. A tool to calculate the amount of rain is described in Lievens *et al.*, 2016.

5.4.3 Resolution

Peak resolution is a concept from the field of HPLC (High Pressure Liquid Chromatography) that translates well to dPCR when applied to the density plots of the droplet fluorescence readings. The resolution of a digital assay (R_s) is a quantitative measure of how well the two populations (positive and negative), hereinafter referred to as peaks, can be differentiated in a linear separation. It is

defined as the difference in fluorescence between the two peaks, divided by the combined widths of the peaks:

$$R_s = \frac{2 * (t_p - t_n)}{w_p + w_n} \tag{10}$$

where the subscript *p* indicates the population with the higher fluorescence (positive, as opposed to *n* for the negatives). The variables *t* and *w* are the peak fluorescence and peak width, respectively (Figure 8).

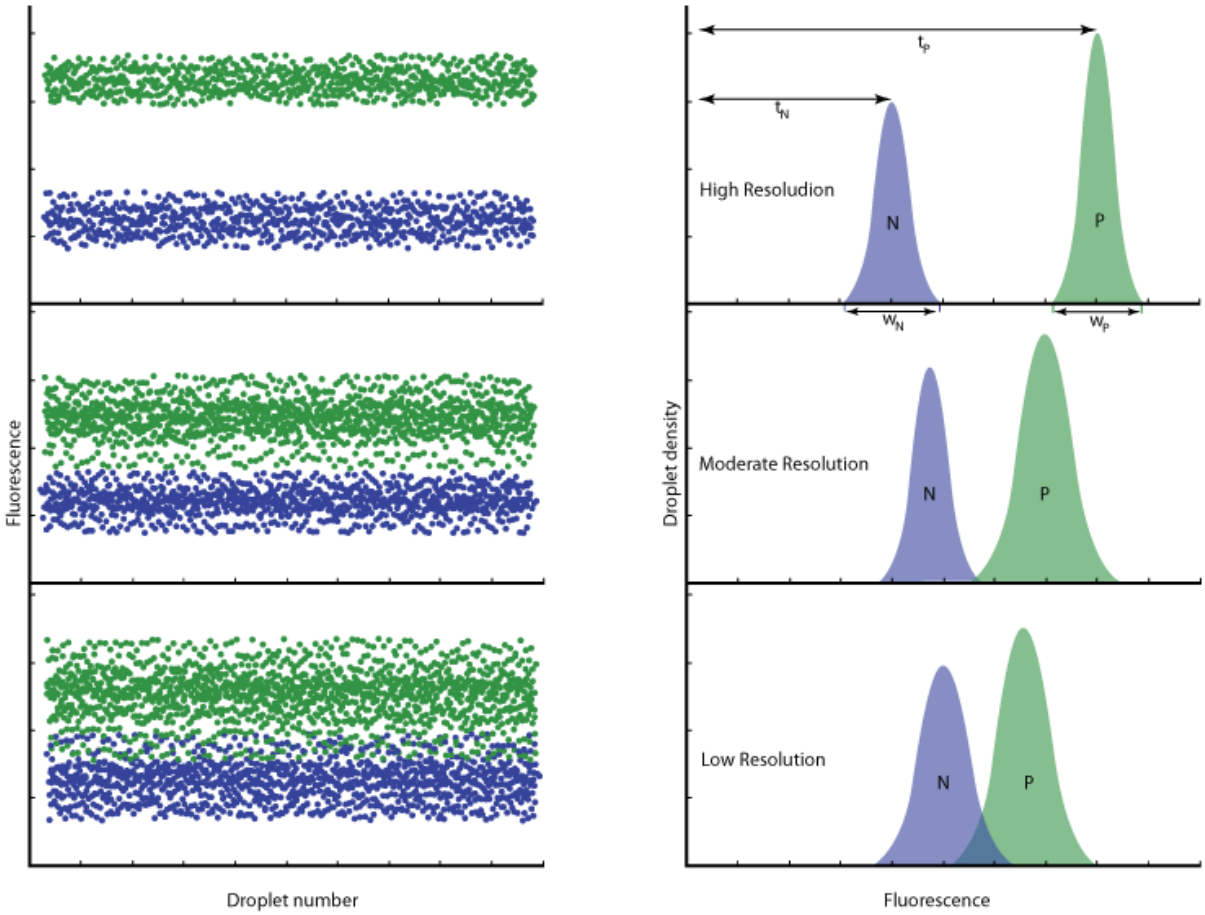


Figure 8: Illustration of the concept of resolution. The left-hand figures show the droplet readout; the right-hand figures show the corresponding density plots. t_n and t_p show the fluorescence positions with the highest density in the negative and positive droplet clouds, respectively. w_n and w_p represent the width of the density peaks at their base (Lievens *et al.*, 2016).

Essentially, the resolution corresponds to how well the fluorescence of the droplet populations is separated. A resolution of 2 represents a complete separation; we propose R_s **= 2** as a minimum to allow for a certain amount of deterioration of the resolution in more difficult samples. At R_s **= 2.5** there is high resolution and at R_s**= 0.5** there is low resolution.

5.4.4 Target concentration

As mentioned in several papers (Dube *et al.*, 2008; Jacobs *et al.*, 2014), there is an optimal concentration of target molecules per partition that should yield the least random sampling variability (and thus the narrowest confidence bounds). This optimal value of λ for absolute quantification is approximately 1.6, i.e. if random sampling variation as described by the Poisson

process is the only source of variability. For a partition size of 0.85 nl (ddPCR), this translates to an optimum of about 1,870 target copies per µl or 37,400 per 20 µl total reaction mix. For a partition size of 6 nl (cdPCR), this translates to final concentrations of about 265 copies per µl or 2,650 copies per 10 µl total reaction mix for optimal conditions.

An upper and lower bound for optimal quantification can be found by inspecting the fraction of reactions that have more than 25 % error due to Poisson variation (see Lievens *et al*., 2016 for details on the bootstrap approach). Table 1 shows the results for the bootstrap. In addition, the table also shows how these limits shift when instead of a single reaction, multiple repeats are used (i.e. averaged) to estimate λ.

Table 1: Results from the bootstrap analysis The table lists the lowest and highest values (as λ and as number of target copies per reaction) at which 95 % of all reactions contain less than 25 % error.

dPCR system	20,000 partitions				765 partitions			
Value	λ		DNA copies		λ		DNA copies	
repeats	lower	upper	lower	upper	lower	upper	lower	upper
1	0.0030	8.86	61	177,297	0.077	5.51	59	4,218
2	0.0014	9.51	29	190,270	0.038	5.31	29	4,063
3	0.0012	9.92	23	198,378	0.030	5.31	23	4,063
4	0.0011	10.00	21	200,000	0.020	6.53	15	4,993

5.4.5 Amount of sample compartmentalized

Both droplet and chamber-based dPCR systems are subject to variability in the number of partitions that are generated and/or accepted into the analysis. On top of that, most compartmentalization techniques have a certain 'dead volume'. Consequently, and unlike in qPCR, the entire volume of sample loaded into the chamber/droplet generator is not analysed. This essentially corresponds to a form of subsampling which may in turn add variation or error to the quantification, especially for reactions with targets at very low abundance. We can express the number of partitions in the analysis in a relative way, i.e. as the fraction of sample compartmentalized: the total volume of partitions accepted into the analysis divided by the total volume loaded into the device.

For droplet-based dPCR, (in silico) simulation results show that there is an increase in variability as less of the total sample is compartmentalized (Lievens *et al*., 2016). However, for endogene λ = 3 and 1 % analyte, one can go down to very low levels of compartmentalisation (< 10 %) before the chance of excessive error (> 25 %) becomes greater than 1 in 20 (i.e. 95 % of the simulations have less than 25 % relative error). For lower levels of analyte, a higher compartmentalisation level is required to keep 95 % of the simulations below 25 % error, and the same is true for lower values of λ. From these results, we propose to require at least 30 % compartmentalisation for quantification down to 1 %, and 50 % compartmentalisation for quantification down to 0.5 %. Runs with lower compartmentalisation should therefore be omitted.

5.4.6 Unit of measurement

The GM content measured by dPCR is expressed as a copy number ratio but does not provide an estimate of the amount of GM in mass fraction as required by the EU legislation. The conversion of copy number ratio into mass fraction can be done via one single conversion factor (CF) per event. A

recent guidance document explains how to report traceable and comparable results expressing GM content in accordance with EU legislation (Corbisier *et al.*, 2017).

5.5 Data interpretation and reporting

5.5.1 Data interpretation

As far as data interpretation is concerned, a dedicated ENGL Working Group on "Detection, Interpretation and Reporting on the presence of authorised and unauthorised genetically modified materials" (WG DIR) has been established in order to amend and update the previously produced document on "The detection, interpretation and reporting on the presence of unauthorised genetically modified materials" (Ciabatti *et al.*, 2017). This document is designed:

- To help laboratories to take the appropriate decisions on compliance on the basis of the results obtained and the requirements of the EU legislation on GM food and feed currently in place;
- To provide all the relevant data and information on analytical reports according to the obligations of the EU legislation on GM food and feed and ISO standards.

Even though the previous document is non-exhaustive, it addresses the most frequent cases and should be considered as a source of guidance on the matter. It applies to the official analytical control of the following GM events for the enforcement of EU legislation on GM food and feed. Some particular cases may require further adaptive interpretation. Future developments and/or legal requirements (e.g. new tolerance or labelling thresholds, etc.) should be taken into consideration in case of future amendments to the document.

- GM events authorised on the EU market for food and feed: for traceability and labelling requirements, the laboratory is requested to verify the presence of these events above a 0.9 %-threshold;
- GM events unauthorised or withdrawn from the EU market, which are falling under the scope of Reg. (EU) 619/2011: the laboratory is requested to verify the presence of these GM events taking into consideration the Minimum Required Performance Limit (MRPL) set by the Regulation at 0.1 %. A technical guidance document on the implementation of Commission Regulation (EU) 619/2011 has been developed and published by the EURL GMFF (Mazzara *et al.*, 2011);
- GM events unauthorised or withdrawn from the EU market, to which a Commission Implementing Decision on the withdrawal from the market and on a tolerance period for traces applies. The laboratory is requested to verify the presence of these GM events taking into consideration the MRPL of 0.1 %. For GM events currently fulfilling the requirements of Reg. (EC) No. 619/2011 as well as for information about specific validated detection methods and the source of certified reference material please refer to: http://ec.europa.eu/food/dyna/gm_register/index_en.cfm.
- GM events unauthorised or withdrawn from the EU market, which are not falling under the scope of Reg. (EU) 619/2011 and to which no Commission Implementing Decision on the withdrawal from the market and on a tolerance period for traces applies: the laboratory is requested to verify the presence of these GM events without any tolerance threshold or any MRPL.

The WG DIR document does not address the official analytical control of seeds, for which no traceability/labelling threshold or MRPL has yet been established in the EU. However, the reporting requirements are also applicable to seed testing.
A decision tree has been presented by the group and refers to the following stepwise analytical work flow, which is generally adopted by laboratories to verify the enforcement of EU legislation on GM food and feed:

1. Detection of the ingredient/component/constituent of the food/feed: this is performed using taxon-specific methods;
2. GMO screening: this is usually performed using element and/or construct-specific methods;
3. GM event identification using qualitative event-specific methods;
4. GM event quantification using quantitative event-specific methods.

However, there may be specific cases where one or more of the steps listed above are not necessary or useful and may be skipped. For unauthorised GMOs (UGMs), step 3 and 4 will often not be possible because of the lack of event-specific detection methods and/or reference materials. Zero tolerance of UGMs in food is prescribed in the EU (Figure 9).

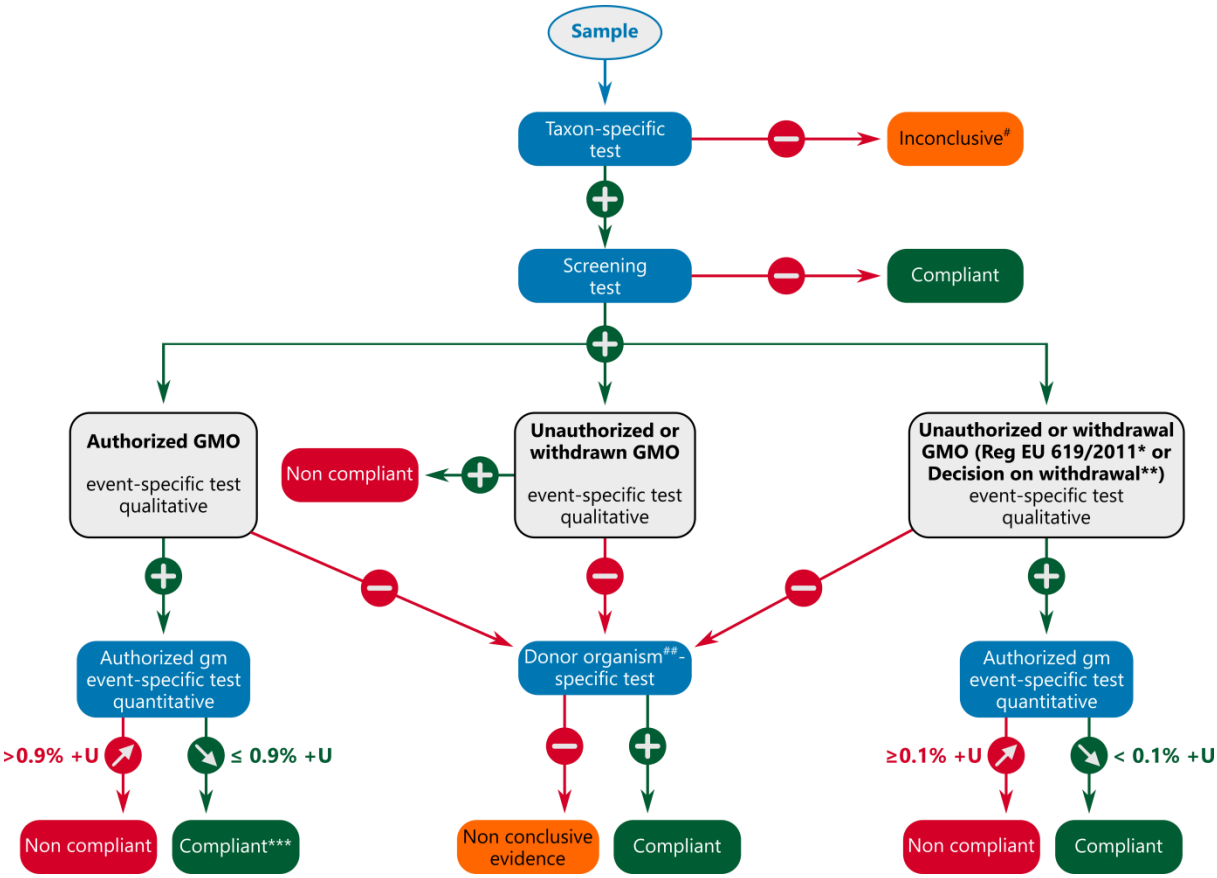


Figure 9. Decision tree for GMO analysis (Figure based on Ciabatti *et al.*, 2017).

The term "inconclusive" is used in this context with the meaning that, if a taxon is not detected, the analytical request to detect and identify possible GM events for this taxon cannot be satisfied.

The term "donor organism-specific test" means to test for the presence of organisms in which the screening elements are naturally present.

* 0.1 % Minimum Required Performance Limit applies to methods for the detection of GM events falling under the scope of Regulation (EU) No 619/2011

** A tolerance threshold applies to some products according to specific Commission Implementing Decisions on their withdrawal from the market: e.g. Commission Implementing Decision (EU) 2016/2268 of 14 December 2016 amending Decisions 2007/305/EC, 2007/306/EC and 2007/307/EC as regards the tolerance period for traces of Ms1xRf1 (ACS-BNØØ4-7xACS-BNØØ1-4) hybrid oilseed rape, Ms1xRf2 (ACS-BNØØ4-7xACS-BNØØ2-5) hybrid oilseed rape and Topas 19/2 (ACS-BNØØ7-1) oilseed rape, as well as of their derived products; it sets a 0.1 % tolerance threshold until 31 December 2019.

*** provided that this presence is adventitious or technically unavoidable

Results should be reported according to ISO 24276/Amd1:2013, ISO 21569/Amd1:2013 and ISO 21570/Amd1:2013

5.6 Digital MIQE guidelines

The growth of interest in dPCR, both as an aid in metrological traceability and as a real-life application across a range of sectors including food testing, means that a plethora of data is being produced. This has led to the establishment of a set of guidelines for the production and publication of dPCR data, in order to harmonise the approach and provide meaningful results which can be readily interpreted (dMIQE guidelines, Huggett *et al*., 2013). Methods based on dPCR can be applied in various areas of food analysis. The methodology is a quantitative technology by nature, but it can be used for screening purposes as well.

The original qMIQE guidelines (Minimum Information for Publication of Quantitative PCR Experiments; Bustin *et al.*, 2009) were published with the aim of improving qPCR analyses and ensuring data comparability and reproducibility. These were rapidly adopted by a number of stakeholders as a useful way in striving towards harmonisation of qPCR results.

Shortly after this in 2013, the digital MIQE guidelines (dMIQE; Minimum Information for Publication of Quantitative Digital PCR Experiments guidelines; Huggett *et al.*, 2013) were published, in order to help harmonise results from dPCR experiments. The dMIQE guidelines provide a checklist of items to address when publishing results from dPCR experiments to help ensure that results can be reproduced, followed and understood. The aim of the dMIQE guidelines is three-fold: i) to facilitate replication of experiments; ii) to provide critical information to allow analysts and reviewers to measure the technical quality of work; iii) to help towards harmonisation of reporting of results/comparison of results irrespective of dPCR instrumentation used.

The items in the dMIQE guidelines checklist are categorised as either essential (E) or desirable (D) to report when publishing results.

Essential (E) information to report includes, but is not limited to:

- Mean DNA target copies per partition (lambda);
- Number of partitions used (lambda and the partition number determine the precision associated with the experiment);
- Template structural information – there is evidence to suggest that the nature of the sample can affect the reliability and accuracy of the result. It is important to capture aspects of the structural information of the template, including: template type (e.g. genomic, plasmid);

source (e.g. organism, tissue, cell, flour, food, plant, leaf); treatment (e.g. restriction digestion, sonication, pre-amplification, none);
- Individual partition volume – different dPCR platforms have different partition volumes[2];
- Total volume of reaction = (number of partitions) x (partition volume). This is particularly important for ddPCR as total volume can differ between runs because of variability in number of partitions generated;
- Nature of controls used;
- Supplemental data – representative amplification plots (or endpoint fluorescence values) of positive and negative experimental results;
- Example experimental variance – the analyst is encouraged to take multiple biological replicates to more accurately capture the experimental uncertainty.

Desirable (D) information to report includes, but is not limited to:

- Optimisation data for the assay;
- Total initial PCR reaction volume prepared – not all instruments use the total volume and sample volume may be much smaller;
- LOD of assay.

It is a recommendation of this WG that the essential items outlined in the dMIQE guidelines should be documented when publishing dPCR experimental results, as an aid to harmonised presentation of results from analysis of GMOs using dPCR instrumentation.

6. Costs and practicability of dPCR

Assuming that all currently active GMO laboratories already possess qPCR equipment, the laboratory may benefit from performing a cost-benefit calculation prior to investing in dPCR equipment.

6.1 Instruments costs

Currently two types of technology are applied to create reaction partitions. One is based on specially designed chips or plates (Fluidigm, Formulatrix or Applied Biosystems) and the other sequester reagents into individual droplets (Bio-Rad, RainDance and Stilla Technologies). Recent advances in nanofabrication and nanofluidics enabled building dPCR platforms capable of generating thousand or even millions of PCR partitions. The final costs of analysing each sample will depend greatly on the platform, price of consumables and the time required to set up and analyse samples.

6.2 Consumables cost

The price for consumables as well as for instruments can vary depending on the producer's pricing policy for a particular market. Digital PCR uses the same reagents (primers and probes) as qPCR but specific consumables (chips, cartridges, gaskets, holders, pipettes etc.) are required for each instrument. For ddPCR, the QX 100/200 ddPCR system from Bio-Rad can generate up to 20,000 droplets (usually 14,000–18,000) per sample on an 8-sample cartridge. Up to 96 samples can be analysed at once assuming samples from 12 cartridges are transferred into a 96-well PCR plate and run together. The Naica System is based on chips that can be used for four samples. Each sample is distributed into approximately 30,000 individual droplets, where a maximum of three chips can be run simultaneously. RainDrop Digital PCR can generate up to 10,000,000 droplets per sample and run

[2] also see Vynck and Thas, 2018

8 samples per chip. For cdPCR, Fluidigm offers partition of samples into 765 chambers in each of the 12-chip panels (765 chambers x 12 panels = 9,180 reactions per chip), or a chip with 48 arrays (770 chambers per array) capable of producing 36,960 individual PCRs simultaneously. The QuantStudio 3D uses one chip per sample, where the reaction mixture is distributed into 20,000 wells present in a chip. Twenty-four chips can then be run together in a PCR cycler, but each chip needs to be read individually. The Constellation system from Formulatrix offers plates where 96 samples can be loaded and each is distributed into 8,000 chambers or 24 samples can be loaded and each is distributed into 36,000 chambers.

6.3 Practicability

Each dPCR platform has some advantages and limitations in terms of using it in practice. There are also some bespoke procedures to learn in order to generate the required number of droplets or fully load the chip. Some systems enable analysis of the full kinetics of the amplification per partition (BioMark HD, QuantStudio 12K Flex), whilst others are built to detect end-point amplification only (Bio-Rad QX100, QX200, RainDrop Digital PCR, Naica System, QuantStudio 3D, Constellation).

When comparing qPCR and dPCR in terms of costs, some producers of dPCR platforms advertise the systems on the basis of very low costs for a single data point. This price can be very small especially in systems capable of generating millions of partitions. However, the instrument should be tailored to each application which may require a large number of 'single data points' to generate a meaningful result. For instance, two hundred chambers are sufficient to distinguish between 2 and 3 copies in a sample, but at least 8,000 partitions are required to distinguish between 10 and 11 copies. Systems generating millions of partitions are capable of detecting very rare mutations or quantifying very low concentrations of DNA copies, as with more partitions a higher dynamic range can be achieved.

It is therefore difficult to estimate the exact costs of using dPCR in routine testing or research as the total price will depend on several factors. Each user should estimate the costs of dPCR in particular applications taking into account:

- The price of the instrument and costs of maintenance;
- Costs of consumables and reagents;
- The achievable throughput of the system;
- Ease of use of the system;
- Required precision and sensitivity;
- Possibility of multiplexing;
- Additional capacity of performing qPCR;
- Hands-on time;
- Sample throughput per runtime;
- Analysis time.

The costs for analysing a sample with dPCR and qPCR can be comparable or deviate strongly depending on the different platforms that are compared. This comparison only makes sense within a particular market which is being considered (and not between different/deviating markets). The aspect of hands-on-time for analysis and the complexity of handling are crucial parameters that need to be considered.

The qPCR technology has been present on the market for around two decades. As a result, it is well established in many testing and research labs. It has higher throughput and for many routine applications it also costs less. Digital PCR can offer more precision and less uncertainty than qPCR, however, it offers a narrower dynamic range. Some dPCR systems that require less hands-on time will probably find more application in routine testing, especially in medical diagnostics where reproducibility is of great importance. Other systems that offer more flexibility might become the system of choice in research labs.

In case of GMO quantification, dPCR offers similar performance as qPCR but in some cases can outperform qPCR in terms of cost-effectiveness. The possibility of multiplex quantification of GM events of the same species is a good example of reduced hands on time and costs compared to qPCR quantification. A method for direct quantification of twelve approved GM maize lines with multiplex assays with ddPCR recently (Dobnik *et al.*, 2015) proved to be cost-efficient particularly for testing higher number of samples. Direct quantification of twelve approved GM maize lines with 4-plex and 10-plex multiplex assays with ddPCR compared to direct quantification of twelve approved GM maize lines in simplex reactions with qPCR proved to be 3 times cheaper and can save six working days until the result is obtained (the case of analysing 11 samples). For labs testing many mixed samples, the most cost efficient approach could be combining qPCR screening of all samples prior to multiplex ddPCR quantification of the screening positive samples. Developed ddPCR multiplex maize assays can provide more cost-effective GMO quantification, especially when the system is extended to include additional species relevant GM events. In case of quantification of various targets as one group, dPCR offers high flexibility of multiplexing and can easily be applied to diverse testing fields.

6.4 In-house verification

For the in-house verification of dPCR methods (based on validated qPCR methods which are to be transferred to a dPCR format), several parameters associated with quantification should be inspected. Analogous to qPCR, these parameters are: specificity, trueness (when possible), repeatability, robustness, and LOD/LOQ (Hougs *et al*., 2017). The variability associated with the repeatability, intermediate precision and bias can be used to estimate the measurement uncertainty of a dPCR result. The general principles provided in the technical report from Trapmann *et al.* (2009) to estimate the analytical variability of quantitative analytical results obtained by real-time PCR can also be applied for dPCR methods. The approach detailed below to determine the uncertainty associated to a dPCR result is using data derived from within-laboratory samples (ISO 5725-2; Linsinger, 2008).

6.4.1 Specificity

The dPCR method does not differ in its concept from qPCR. The principle of amplification of DNA target molecules is the same as for qPCR. The specificity of a PCR assay is provided by the use of high-fidelity polymerase and an appropriate primers/probe design (DNA sequences). If the specificity of a method has been demonstrated in qPCR, the sequence of the primers/probe as well as the reaction conditions should be maintained in a dPCR format. Changes may have to be made to optimise dPCR (e.g. by altering $MgCl_2$ concentration of the PCR reaction buffer, by changing the annealing temperature, the primer and probe concentrations or the ramping setting). Depending on the changes made to the method (e.g. master mix, thermal program, etc.) a re-evaluation of the method's specificity may be necessary (see 4.3 and annex B.3 for details).

6.4.2 Repeatability

Repeatability is the precision under a set of repeatability conditions that includes the same measurement procedure, same operators, same measuring system (method and dPCR device), same operating conditions and same location and replicate measurements on the same or similar samples over a short period of time. To estimate the repeatability, one can use for example two samples, a high concentration (e.g. 10 % GM material) and a low concentration (e.g. 1 % material), which are analysed in triplicates across five different days. The results are then inspected for their variability over the different days, e.g. by a one-way ANOVA (Analysis of Variance), an example of which is given below.

With the three replicates over the five days there are five estimates of the variance. The MS_w (within Mean Square) captures the variability within each different day. The relative repeatability standard deviation (RSD_r) is given by equation 11:

$$RSD_r = \frac{\sqrt{MS_w}}{\bar{y}} \tag{11}$$

where $\bar{y}$ is the overall mean estimate, and MS_w is the within Mean Square expressing the variance observed within a day.

6.4.3 Intermediate precision

The Intermediate precision reflects the precision over time (for example between days). The relative between-day standard deviation RSD_{ip} is given by equation 12:

$$RSD_{ip} = \frac{\sqrt{\frac{MS_b - MS_w}{n}}}{\bar{y}} \tag{12}$$

where MS_b is the between group variance capturing the variability between each days, MS_w is the within Mean Square capturing the variance observed within a day, n is the number of replicates per day and $\bar{y}$ is the overall mean estimate.

When MS_w is bigger than MS_b the maximum hidden between-day-variation (s^*_{ip}) can be calculated with equation 13:

$$s^*_{ip} = \sqrt{\frac{MS_w}{n}} * \sqrt[4]{\frac{2}{p\,(n-1)}} \tag{13}$$

where, MS_w is the within Mean Square capturing the variance observed within a day, n the number of replicates per day and p the number of days.

The relative standard deviation for intermediate precision (RSD_{ip}) is then obtained by dividing s^*_{ip} by the overall mean estimate $\bar{y}$ (equation 14).

$$RSD_{ip} = \frac{s^*_{ip}}{\bar{y}} \tag{14}$$

Note: In this example, the experiment is repeated on different days to capture the day-to-day variation, however it also possible to perform several runs on one single day to cover all factors that may contribute to the uncertainty of the measurement.

6.4.4 Standard relative uncertainty related to precision

From the above, one can calculate the relative standard uncertainty related to the precision within one laboratory by combining the relative standard deviations associated with repeatability and intermediate precision, as shown in equation 15:

$$u_{precision,rel} = \sqrt{\frac{RSD_r^2}{n \times p} + \frac{RSD_{ip}^2}{p}} \tag{15}$$

where $u_{precision,rel}$ is the relative standard uncertainty related to the precision, RSD_r the relative repeatability standard deviation, RSD_{ip} the relative standard deviation for intermediate precision, p the number of days and n the number of replicates per day.

Note: The uncertainty related to the conversion of the number of positive droplets into copy number concentration varies very much in relation to the lambda value. The lowest uncertainty is observed for a lambda around 1.6 (Lievens *et al.*, 2016). This means that the solution with the extracted DNA for the endogen assay needs to be diluted before being added to the master mix, whereas in most cases the DNA of the transgene does not need to be diluted.

6.4.5 Trueness

Definition: Closeness of agreement between the average value of an infinite number of replicate measured quantity values and a reference quantity value. The measure of trueness is usually expressed in terms of bias (ISO/IEC Guide 99:2007). The bias is the difference between the expectation of the test results and an accepted reference value (ISO 5725-1:1994). The absence of significant bias should be tested over the whole dynamic range of the method.

Note: An accepted reference value is a value that serves as an agreed-upon reference for comparison, and which derives from:

a) a theoretical or established value, based on scientific principles;
b) an assigned or certified value, based on experimental work of a national or international organisation;
c) a consensus or certified value, based on collaborative experimental work under the auspices of a scientific group;
d) the expectation of the (measurable) quantity, i.e. the mean of a specified population of measurements – when a), b), c) are not available.

The trueness of a result obtained by dPCR can be assessed by comparing the average measured value of a CRM with its certified value. The CRM should be a material certified for its absolute copy number concentration or a material certified for its copy number concentration ratio. The bias between the measured concentration ($Conc_m$) and the certified copy number concentration ($Conc_c$) is provided in equation 16.

$$bias = |Conc_m - Conc_c| \tag{16}$$

The uncertainty associated with this bias can be calculated as followed:

$$u_{bias} = \sqrt{u_{precision}^2 + u_{CRM}^2} \tag{17}$$

where u_{CRM} is the uncertainty of the certified value obtained by dividing the expanded uncertainty (U_{CRM}) by a coverage factor *k*, both found on the certificate of the CRM. The standard uncertainty related to precision ($u_{precision}$) is calculated when the relative standard uncertainty related to precision ($u_{precision,rel}$) – provided by equation 15 – is multiplied by the mean value ($\bar{y}$) obtained when analysing the CRM.

$$u_{precision} = u_{precision,rel} * \bar{y} \tag{18}$$

The expanded uncertainty U_{bias} is estimated by multiplying the standard uncertainty associated to the bias u_{bias} by a coverage factor *k* = 2, providing a level of confidence of approximatively 95 %.

$$U_{bias} = 2 * u_{bias} \tag{19}$$

Acceptance criterion: A bias will be significant if it is larger than the expanded uncertainty of the difference between result and certified value (U_{bias}) [ERM application note 1, 2010]. If a significant bias is observed, either the origin of the bias should be further investigated (*e.g.* through improvement of the dPCR method) or the results should be corrected for that bias.

6.4.6 Uncertainty estimation

The relative uncertainty of a measurement result ($u_{meas,rel}$) can be estimated by combining the relative uncertainty associated with the precision ($u_{precision,rel}$) with the relative uncertainty associated with the trueness ($u_{bias,rel}$). The uncertainty of the bias is included in the uncertainty budget regardless of whether or not the bias was found to be significant (Linsinger *et al.*, 2008).

$$u_{meas,rel} = \sqrt{u_{precision,rel}^2 + u_{bias,rel}^2} \tag{20}$$

$u_{bias,rel}$ is obtained by dividing u_{bias} (Eq. 17) by the mean value measured on the CRM.

Note: The relative standard uncertainty for calculating uncertainty estimates can be used when the percent coefficient of variation is approximately constant over a substantial portion of the measuring range (in our case between 1 % and 10 % GM material).

General note: $u_{x,rel} = \frac{u_x}{\bar{y}}$ or $u_x = u_{x,rel} * \bar{y}$

where $u_{x,rel}$ is the relative standard uncertainty, u_x is the standard uncertainty and $\bar{y}$ the mean value. The standard uncertainty is expressed in the unit of measurement (e.g. in g/kg, GM % or DNA copy number ratio). The relative standard uncertainty is expressed as a percentage relative to the mean value.

6.4.7 Limit of Detection (LOD), Limit of Quantification (LOQ)

The limit of detection (LOD) is the lowest amount or concentration of analyte (e.g. DNA) in a sample which can be detected – but not necessarily quantified – based on a given probability. Experimentally, methods should detect the presence of the analyte for at least 95 % of the cases (samples) at the LOD, ensuring ≤ 5 % false negative results. Analogous to qPCR, the LOD can be established in dPCR by measuring successive dilutions of the analyte in replicate measurements.
The measurement principle of the dPCR makes it possible to detect down to one copy of a target sequence and the Poison distribution is applicable at these very low copy number concentrations. Therefore, it is reasonable to assess the LOD (and LOQ) by performing many replicate measurements at very low copy number concentrations. The assessment should start by clearly defining the level of confidence appropriate for the intended use of the method. When performing an experiment e.g. with 60 replicate measurements (Marchesi *et al.*, 2015) exactly at the LOD with a confidence interval of 95 % there would be on average three negative measurement results, as this represents 5 % of the cases. If all of the 60 replicate measurements performed at a certain PCR copy number concentration are positive, it can be reasonably assumed that this concentration is above the LOD of the method (Deprez et al. 2016). As this approach may not always be feasible, a pragmatic approach based on a lower number of replicate measurements could be followed for the verification of the LOD. This approach allows an approximate estimation of the LOD. Dilution series representing the range above and below the expected LOD, based on prior knowledge of the LOD of that method (e.g. from validation data), are tested in e.g. a minimum of 10 PCR replicate measurements for each concentration level. The lowest concentration where all replicate measurements are positive is the estimated LOD (Hougs *et al.*, 2017). The use of a different approach should be supported by sound statistical evidence ensuring that the level of confidence required is reached (Marchesi *et al.*, 2015).

The LOD (and the LOQ) of a dPCR method depend on the number of analysed partitions and the total volume of the analysed partitions. It is therefore possible to calculate the theoretical minimum LOD for a given dPCR set-up based on the Poisson distribution. The calculation is based on the combination of two probabilities: the probability of pipetting the DNA copies in the mix (*sampling*) and the probability that these DNA copies end up in the droplets. In the case of a ddPCR with e.g. 17,000 analysed (accepted) droplets with an assumed droplet volume of 0.85 nL (also see note of table A. 1b in the annex), the minimum theoretical LOD will be 0.29 cp/µL in the PCR mix. At this concentration level, 5 % of the measurements will not have a single copy of the target sequence in the analysed droplets.

The Limit of quantification (LOQ) is the lowest amount or concentration of analyte in a sample that can be reliably quantified with an acceptable level of trueness and precision. This means that the LOQ of a dPCR method depends on the level of uncertainty considered acceptable given the intended use of the method. Practically, the LOQ of a dPCR method can be established by measuring the relative uncertainty of a measurement result ($u_{meas,rel}$) at a low copy number concentration or ratio.
For some dPCR platforms, the manufacturer may give indications about the lower bound of the number of partitions needed for accurate quantification.

6.4.8 Robustness

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

Acceptance criterion: the method should provide the expected results when small deviations are introduced from the experimental conditions described in the procedure.
For quantitative modules, based on the acceptance criterion of ≤ 25 % for the relative repeatability standard deviation (RSDr) and trueness, the RSDr and trueness calculated for a combination of changes should not exceed 30 % (Marchesi *et al.*, 2015).

Examples of factors that a robustness test should address are experiments performed by another operator, different primer and probe concentrations, different annealing temperatures, different thermal cyclers, different reaction volumes, and different master mix suppliers (for examples on how to assess robustness see e.g. Marchesi *et al.*, 2015; Hougs *et al.*, 2017).

6.5 Accreditation

A dPCR method can be submitted for accreditation under ISO 17025. The process of submission strongly depends on the scope of the accreditation. Before submission, it is advised that the national accreditation body is consulted regarding the dPCR method submission. Digital PCR is essentially just a variation of PCR, using the same primers/probes (and in some cases master mix) as qPCR. Documents for method submission should be prepared in accordance with a quality system. If a laboratory has a scope for PCR based methods, general documents could just be complemented with description of dPCR and new documents relating to the dPCR procedures should be prepared. A dPCR method must fulfil the above-mentioned verification criteria, if the assays were transferred from qPCR to dPCR. New methods should be validated, and pass all of the acceptance criteria if they are to be considered for accreditation.

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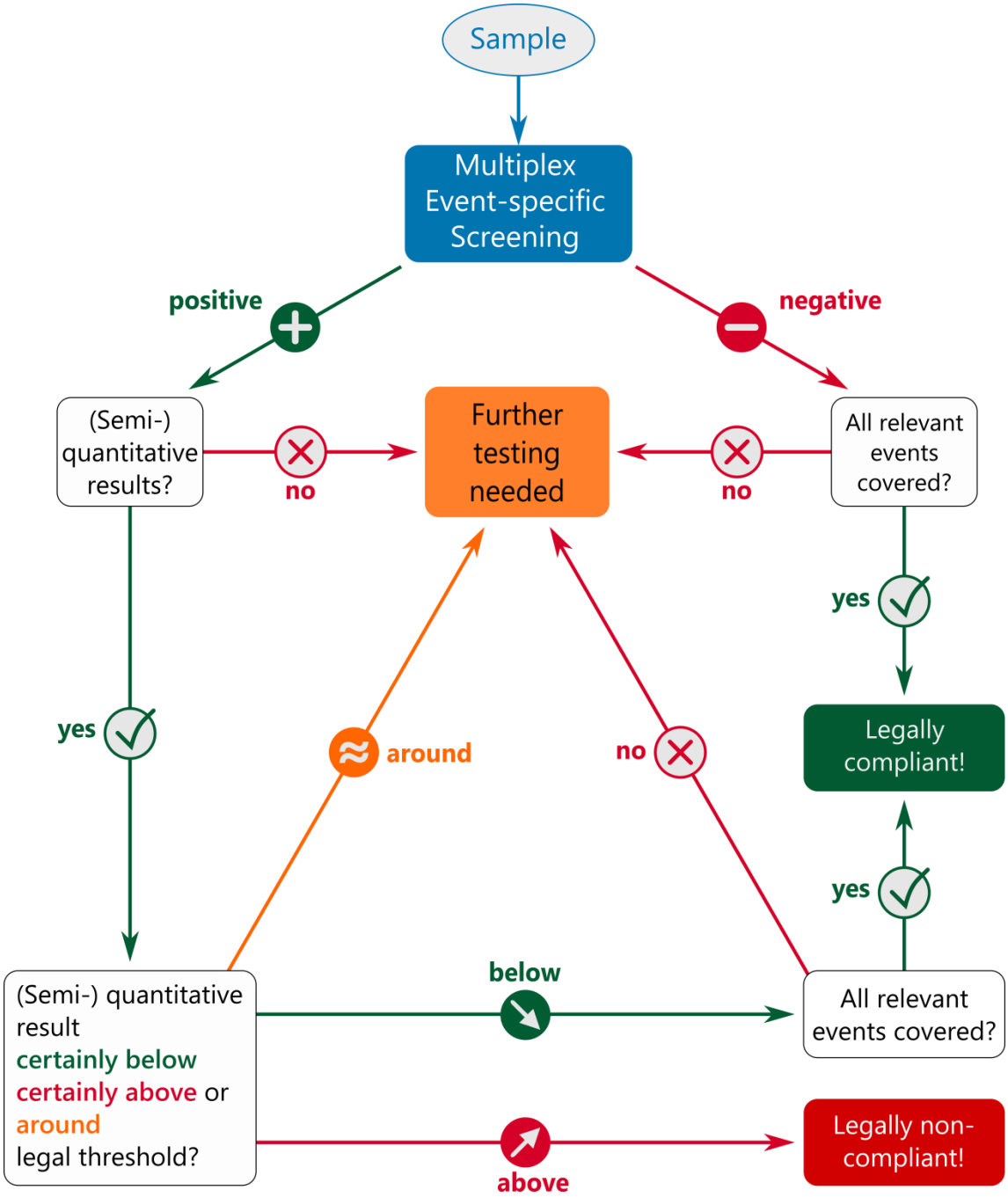
Annex

A. Application of dPCR in the field of GMO detection and quantification – explanations, notes and examples

A. 1 Flow diagram/decision tree for application of (multiplex) event-specific screening methods for GMO detection



Event-specific (multiplex) screening approaches (see main text for examples from literature) can be used to reach preliminary or conclusive decisions. *Positive* and *negative* refers to signal detected and signal not detected. Further testing refers to optional use of e.g. singleplex event-specific methods for identification and/or quantitation. Relevant events refer to events covered by the scope of the test, e.g. in the case of a soybean product it could be: 1) all EU-authorised soybean events in case of

testing for compliance with the authorization and labelling regulations 1829/2003 and 1830/2003; or 2) all soybean events under LLP regulation in the EU in case of testing for compliance with Regulation EC 619/2011, respectively. Legally compliant and non-compliant refers to cases where the product is or is not compliant with the legal requirements in e.g. one of the mentioned regulations. Certainly above or below a legal threshold refers to the inferred concentration/value after taking the associated measurement uncertainty into account.

A. 2 Lambda optimised approach

The dynamic range of a dPCR may cover four to six orders of magnitude, approaching those of qPCR (up to seven orders of magnitude), depending on the partition number of the dPCR device used (RainDance Technologies device; Jones *et al.*, 2016). It should be mentioned that some devices/analysis tools allow several wells to be merged in order to increase the number of partitions analysed and thus to increase the dynamic range. However, as described for chamber dPCR (BioMark device; Bhat *et al.*, 2009) or droplet dPCR (Bio-Rad device; Pinheiro *et al.*, 2012; Deprez *et al.*, 2016), the relative uncertainty of the determined concentration is not constant across the dynamic range. The stochastic effect (i.e. impact on measurement uncertainty) associated with the (sub-)sampling (* see note) of a DNA solution increases as the concentration of the DNA solution decreases. It is advisable for dPCR to check the copy numbers of a DNA solution in a test run in order to determine reference and transgene copy numbers. Alternatively, multiple dilutions of DNA can be analysed simultaneously. For samples with low GM content (** see note), i.e. where a low number of transgenic copies are present among a high number of copies of species reference gene, it is advised to use at least two different DNA dilutions.
DNA copy number concentration adjustments can be achieved by testing the DNA extracts at two dilutions, one optimised dilution for the reference gene reaction and one optimised dilution for the transgene reaction. This optimisation avoids having either too many negative or positive partitions for both transgenic and reference gene reaction.

The DNA copy number determination can be done either by simplex or by duplex dPCR, depending on the laboratories´ preference. The result is then determined by combining the results of the two "lambda optimised" reactions taking the dilution factors of the DNA solutions used into account.

* Note: The term ´sampling´ refers to the fact that not the entire PCR reaction (including the DNA molecules) is read in the droplet reader (´dead volume´).

** Note: For the present Bio-Rad System (QX100 or QX200) low transgenic DNA copy numbers would correspond to approximately 0.1 %. For other devices, it could be higher or lower depending on the number of partitions used.

Example for a ddPCR device with a maximum of 20,000 droplets generated:

A maize sample containing 0.1 % (m/m) of MON 810 (hemizygous for the transgene), that sample – assuming a conversion factor (zygosity) of 0.5 – contains 2,000 times more species specific DNA copies (*hmg*) than MON 810 transgene copies. Considering a DNA solution (with 5 µL of that solution in a total dPCR volume of 22 µL) containing 8,282 copies (*** see note) of *hmg* per µL DNA solution and (theoretically) more than 4 copies of MON 810 (2,000 times less). The lambda value (λ = number of DNA copies per droplet in a ddPCR, see 4.1) for the *hmg* reaction will be close to 1.6. At this lambda value, the conversion of positive droplets into copies has the lowest variability and the

dilution applied to the DNA extract is indeed optimal for that *hmg* reaction. However, the lambda value for the MON 810 reaction in this diluted DNA sample will be below 0.001 (Table A 1a, No 1), which is not optimal considering the error due to Poisson variation (Lievens et al., 2016). When, for example, measuring a few transgene DNA copies less, the result (GMO percentage) will substantially change (Table A 1a, No 2). When scaling up the DNA quantity (e.g. roughly three or six times) used in PCR, lambda for MON 810 can be improved (Table A 1a, No 3 and 4). The lambda optimised ddPCR results can then be combined (Table A 1b, No 5 and 6).

Table A 1a. Lambda values in ddPCR for a 0.1 % (m/m) heterozygous gm maize material at different DNA sample concentrations

No	ddPCR	+ d	total d	estimated cp in total d ***	λ [cp/d]	DNA diluted sample [cp/µL] ***	Ratio cp/cp [%]	Ratio m/m [%] a)	DNA dilution factor
1	*hmg*	13,568	17,000	27,200	**1.6001**	8,282	0.051	0.103	6.0
	MON 810	14	17,000	14	**0.0008**	4.26			
2	*hmg*	13,568	17,000	27,200	**1.6001**	8,282	0.033	0.066	6.0
	MON 810	9	17,000	9	**0.0005**	2.74			
3	*hmg*	16,869	17,000	82,716	**4.8658**	25,187	0.048	0.097	2.0
	MON 810	40	17,000	40	**0.0024**	12.19			
4	*hmg*	16,999	17,000	165,592	**9.7410**	50,422	0.048	0.097	1.0
	MON 810	80	17,000	80	**0.0047**	24.42			

+ d: positive droplets
cp: DNA copy number
total d: total measured (accepted) droplets
λ = number of estimated DNA copies per droplet [cp/d]
a): a conversion factor (CF) of 0.5 was assumed to convert cp/cp [%] into m/m [%]

Table A 1b. Combination of lambda optimised ddPCR results taking different dilution factors into account

No	ddPCR	+ d	total d	estimated cp in total d ***	λ [cp/d]	DNA diluted sample [cp/µL] ***	DNA not diluted cp/µL ***	Ratio m/m [%] a)	DNA dilution factor
5	*hmg*	13,568	17,000	27,200	**1.6001**	8,282	49,695	0.097	6.0
	MON 810	40	17,000	40	**0.0024**	12.19	24.39		2.0
6	*hmg*	13,568	17,000	27,200	**1.6001**	8,282	49,695	0.097	6.0
	MON 810	80	17,000	80	**0.0047**	24.42	24.42		1.0

*** Note: The average droplet volume was assumed to be 0.85 nL according to Bio-Rad software QuantaSoft (from version 1.6.6.0320 onwards). There is strong evidence that the average droplet volume is actually smaller, ranging from 0.837 nL (Dong *et al.*, 2015) and 0.834 nL (Corbisier *et al.*, 2015) to 0.718 nL (Košir *et al.*, 2017b), the latter volume depending on the cartridges, droplet generator and master mix used. The (assumed) droplet volume has an impact on the calculation of absolute copy number concentration only. For the determination of a copy number ratio, the average droplet volume has no influence on the calculation results.

In such a case, where low DNA copies (e.g. transgene) are to be quantified, the measurement of two different dilutions of the DNA extract should be considered. In the above mentioned (theoretical) example the DNA extract was diluted to optimize the *hmg* reaction (lambda = 1.6). A three to six times more concentrated — i.e. less diluted — DNA extract (Table A 1a, No 3 and 4) will improve the lambda values for the MON 810 reaction (0.0024 and 0.0047, respectively) but will finally saturate the *hmg* reaction (BioRad Bulletin 6407 Rev A; Taylor *et al.*, 2015). In order to optimise the transgenic reaction and to reduce uncertainties at low DNA concentration due to positive droplet count variation, the number of positive droplets should give the result in copies per reaction above the LOQ of a specific method.

A. 3 Practical aspects of multiplex dPCR

1. Multiplex PCR with several probes labelled with the same fluorescent dye may increase the background fluorescence of the negative droplets reducing the distance between the negative cluster and the positive cluster(s) (Dobnik *et al.*, 2015). A reduced discrimination between the clusters may increase the chance of droplet misclassification and may make quantification of low GM contents more challenging. Therefore optimization and validation of the method is necessary, as for any method or approach.
2. The copy number ratio measured by dPCR needs to be converted into a mass fraction. The applied conversion factor (CF) needs to be linked to the calibrant used in qPCR (to obtain comparable and traceable results between qPCR and dPCR techniques). A conversion factor is determined for each certified reference material (CRM) (Corbisier *et al.*, 2017).
3. A multiplex approach for heterozygous GM material (e.g. GM maize), which sums up different GM contents per species, forces use of a generic species CF which may increase the uncertainty of the measurement, if individual CFs deviate significantly from the generic CF. This is particularly relevant, when quantification at concentrations around a legal threshold is performed. In such cases the performance of individual (singleplex) quantification reactions is recommended.

B. Technical part specific for the implementation of dPCR in a laboratory

In this section a summary of technical recommendations, explanations and notes are given, that are specific to dPCR. For other issues, such as acceptance criteria for method performance, the same requirements apply as for qPCR (Marchesi *et al.*, 2015), if applicable.

B. 1 dPCR platform verification

B.1.1 DNA copy number determination

When establishing a new dPCR device in a laboratory, it has to be demonstrated at least once, that the laboratory is able to properly use this device and DNA copy numbers can be determined with sufficient accuracy in terms of precision and trueness. This is ideally done with reference material certified for absolute DNA copy numbers, if available. Alternatively reference material can be used that is certified for DNA copy ratio (e.g. ERM®-BF425c , Meyer *et al.*, 2010) or for mass/mass fraction. A list of certified reference materials is available e.g. at the JRC (https://ec.europa.eu/jrc/sites/jrcsh/files/rm_catalogue_170424.pdf).

B. 1.2 Fraction of sample compartmentalised

There is an increase in variability as less of the total sample is compartmentalized ('dead volume'). However, for endogene λ = 3 (lambda, λ is the average number of targets per partition) and 1 % analyte, one has to go down to very low levels of compartmentalisation/partitioning (< 10 %) before the chance of excessive error (> 25 %) becomes greater than 1 in 20 (i.e. 95 % of the simulations have less than 25 % relative error). For lower levels of analyte, more compartmentalisation is required to keep 95 % of the simulations below 25 % error, and the same is true for lower values of λ. Based on *in silico* simulations (Lievens *et al.*, 2016) it is proposed to require at least 30 % compartmentalisation for quantification down to 1 %, and 50 % compartmentalisation for quantification down to 0.5 %.

Note: Both droplet and chamber-based dPCR systems are subject to variability in the number of compartments that are generated and/or accepted into the analysis. Additionally, most compartmentalisation techniques have a certain 'dead volume'. As a consequence, and unlike in qPCR, not the entire volume of sample loaded into the chamber/droplet generator is analysed. This essentially corresponds to a form of sub-sampling which may in turn add variation or error the quantification, especially for reactions with targets at very low abundance.

B.1.3 Dynamic Range

The dynamic range of the platform should be assessed. This can be done by analysing serial dilution of DNA with known DNA content (e.g. Jones *et al.*, 2016). The dynamic range of a dPCR increases, as the number of partitions analysed increases.

B. 2 Method transfer of qPCR methods into a dPCR format

When a qPCR method is to be transferred into a dPCR format it is recommended to start with the examination of the reaction conditions (primer and probe concentrations; temperature profile for DNA amplification) as described for the validated qPCR method. Nevertheless this is not always possible, e.g. as certain platforms require specific master mixes.

In case the parameters for dPCR are respected, e.g. single target amplification, amount of rain and resolution is acceptable, use the method as such. Otherwise, if the method is to be modified, then take into consideration the following parameters and aspects:

- Annealing temperature
- Primer and/or probe sequence. In case primer/probe sequence are changed, a full validation of such a new method is needed (Marchesi *et al.*, 2015)
- Use certified reference material (if available) to check for accuracy (trueness and precision), ideally certified for DNA copies (range between 10–100,000 per reaction, 6 parallel reactions per dilution)
- If the composition of an alternative master mix is known and comparable to the conditions of the validated master mix, then the re-assessment of specificity is not required. Otherwise, an experimental check on selected targets (e.g. GM targets and taxa) is recommended

B. 2.1 Peak resolution and intermediate signals in ddPCR

The conditions for establishing the following parameters should ideally be around λ = 0.7 (corresponding to 50 % positive and 50 % negative droplets):

B. 2.1.1 Minimal peak resolution (R_s)

The resolution of a digital assay (R_s) is a quantitative measure of how well the two populations (positive and negative) can be differentiated in a linear separation. It is defined as the difference in fluorescence between the two peaks, divided by the combined widths of the peaks:

$$R_s = \frac{2 * (t_p - t_n)}{w_p + w_n}$$

where the subscript *p* indicates the population with the higher fluorescence (positive, as opposed to *n* for the negatives). The variables *t* and *w* are the peak fluorescence and peak width, respectively.

- Minimal peak resolution (R_s) should be at least 2.0, preferably higher than 2.5

B. 2.1.2 Intermediate signals (*rain*)

Intermediate signals (rain) may be defined as the compartments whose fluorescence readings are between the maximal fluorescence of the negative cluster and the minimal fluorescence of the positive cluster.

- Intermediate signals (*rain*) should be less than 2.5 % of the total number of partitions

B.2.1.3 Threshold setting for analysis

Threshold setting for classification of partitions as positive or negative should be set just above the cluster of negative partitions. Although there is no clear consensus, due to practical experience a reaction is usually considered positive, when the number of positive partitions exceeds two (Dobnik *et al.*, 2015).

Note: No template control (NTC) samples, generating only negative partitions, can help in setting the threshold, however, an inspection of all wells/panels is recommended. The fluorescence amplitude of the negatives in individual wells/panels might be slightly higher or lower than NTCs, thus in such cases threshold can be adjusted individually to avoid misclassification of droplets.

B. 3 Optimization of dPCR

B. 3.1 DNA extraction

Some DNA extraction methods might interfere with droplet generation. The impact of the extraction method on downstream workflow should therefore be evaluated once. This can be done by extracting a) a certified reference material, and b) a food sample, with the standard DNA extraction methods applied in a specific laboratory. Some extraction method may show significantly deviating (poor) performance (e.g. in terms of yield and purity) when applied to certain matrices compared to another method. Therefore yield and purity should be checked beforehand and a method should be chosen that gives favoured performance with that matrix.

B. 3.2 DNA pre-treatment

Under certain conditions (e.g. to separate tandem gene copies, to increase random partitioning, to reduce sample viscosity of high molecular DNA solutions) pre-treatment of DNA (e.g. with DNA dependent restriction endonucleases, 4- or 6-base recognition sites) may be required in order to increase accuracy of dPCR measurement. This is to digest the DNA with enzymes (DNA dependent

restriction endonucleases, e.g. with 4- or 6-base recognition sites). It should be checked and verified, that the enzyme used does not cut the target DNA sequence of interest (i.e. the DNA sequence of the PCR generated amplicon). Additionally it is recommended to check the efficacy of digestion step e.g. by gel-electrophoresis.

Note: It should be avoided to expose DNA to sustained elevated temperatures or alkaline solution as the different strands of fully denatured molecules may be divided across different partitions, leading to two positives for a single (double stranded) target copy. As a consequence, if a significant portion of the sample DNA is denatured, the amount of target will be overestimated if the user assumes that only double-stranded DNA is quantified.

B. 3.3 Target DNA concentration

There is an optimal concentration of target molecules per partition that should yield the least random sampling variability. This optimal value of λ for absolute quantification is approximately 1.6. In practice, this value is not always achievable, especially when analysing samples with low target DNA concentrations (see Annex A. 1).

B. 3.4 PCR reaction

- In general, the temperature profile used in qPCR can be directly applied to dPCR. However, one should always check the manufacturer's recommendations for specific requirements. For example, in droplet-based systems, a final step at higher temperature (e.g. 98 °C, 10 min for the Bio-Rad QX100/200 ddPCR systems) may be mandatory to further stabilize the droplets prior to detection with the droplet reader.
- Sometimes it is advisable to run an annealing temperature gradient to find the optimal annealing temperature. In case of co-amplification of a secondary target (multiple clouds of positive droplets in ddPCR) one can increase the annealing temperature in order to generate only one cloud of positive droplets. Lowering the annealing temperature can lead to better separation between positive signals and background noise.
- Primer and probe concentration can be adjusted if necessary. For testing higher and lower primer and probe concentration than given in the original qPCR method protocol can be used. Higher primer and probe concentrations usually increase the intensity of the end-point fluorescence signal and thus allow better separation of the background noise from specific signals, aiding more accurate quantification of the target. If deemed necessary a primer/probe concentration gradient can be run to improve resolution.
- Touch down PCR can also be applied to enhance specificity which can lead to less rain. Possible conditions for annealing are, e.g. 15 cycle at 63 °C and 15 cycles at 60 °C.
- Increasing the number of amplification cycles can reduce intermediate signals ("rain" in ddPCR), e.g. due to late PCR onset caused by partial inhibition. Suggested total cycle numbers are, e.g. 45, 50, 60 or 75).

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