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21 May 2024

CropLife Europe's request for clarifications as regards the JRC report: Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing part 2

Dear Dr. Vincent,

With this letter, we would like to address several outstanding questions and concerns regarding the JRC report "*Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing part 2*" (MPR2). The first section compiles our requests for clarification while the second section lists elements of MPR2 that we believe are not technically feasible. We would appreciate receiving the clarifications from EURL-GMFF at the upcoming annual meeting on 28 May 2024 and/or in a follow up written communication.

Request for Clarification on Guidance

2.1.1 –Practicability section states "*a dPCR method is considered practicable, if the application to another dPCR instrument can be successfully demonstrated. Alternatively, the method developer may demonstrate that the method has been successfully transferred into a real-time PCR format*". Please provide practical guidelines for the scope of this transfer demonstration if the dPCR method is transferred into a real-time PCR format.

2.1.2 Acceptance criteria applicable to DNA extraction modules – Digital PCR is less sensitive to PCR inhibitors. Please confirm that digital PCR dossier submissions using a DNA extraction module previously validated for real-time qPCR can be bridged to and utilised without re-evaluation. Additionally, please confirm if a new DNA extraction protocol is used for a dPCR method validation, the procedure must only adhere to dPCR guidelines.

2.1.3 Acceptance criteria applicable to digital PCR modules. Specificity

What does a "globally representative collection" entail specifically? Perhaps it would be better reworded as a "representative of cultivation areas".

Secondly, for RTPCR, there is a clear indication of the criteria (variability of Cq values not to exceed 1 Cq). For dPCR, this clear distinction is not made. Can you provide more clarification what the "absence of copy number variations" means for dPCR?

2.1.3 Acceptance criteria applicable to digital PCR modules. Specificity – Demonstration of absence of interactions among primers and probes by *in silico* analysis is not defined in the 2015 MPR. Grohmann, et al (2021) is cited in the new guidance regarding this interaction,

however, the primer/probe interaction concern emphasizes real-time multiplex PCR reactions. It is agreed that this is a good laboratory practice. Can you please explain why this is a requirement to be demonstrated for a dPCR reaction?

2.1.3 Acceptance criteria applicable to digital PCR modules. Linearity – The criteria is for 3 runs and 5 concentration levels, each tested in 3 or more PCR replicates. Why does this exceed what is required for qPCR, which is 2 runs and 4 concentration levels?

2.1.3 Acceptance criteria applicable to digital PCR modules. Dynamic Range and Linearity – The “dynamic range should be assessed in terms of trueness and precision, *either* on an absolute concentration level for the single PCR module *or* in relative concentration ratios for combined PCR modules”. (1) Please confirm that it is up to the method developer to determine which single approach to follow for both the dynamic range and linearity assessments. (2) Do acceptance criteria for the slope and R^2 for linearity apply to both absolute copy number as well as relative content (i.e., combined module)?

Annex 1. Recommendations for digital PCR methods. Quality criteria for reactions – Please explain how one determines or measures the rate of intermediate fluorescence (‘rain’) to achieve an accurate estimation of below 2.5%? Can this be assessed and estimated visually with an image provided and is <2.5% a recommendation or a requirement?

Annex 3. DNA modifications prior to dPCR – DNA sonication was excluded in section 2.1.1 (*The use of random fragmentation techniques (e.g., sonication) is not acceptable*), yet it is included as an example in this section. Can you provide a clarification?

Requirements that are not technically feasible

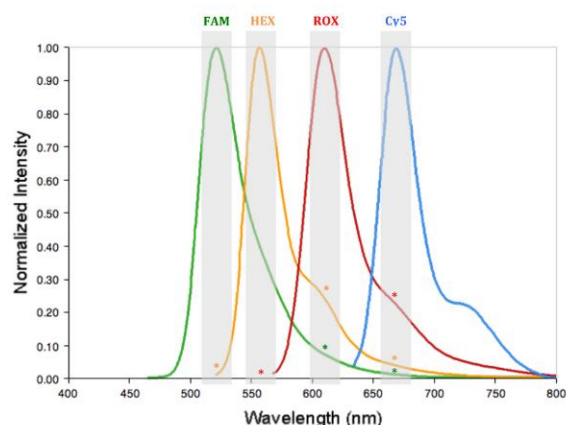
1. Introduction (Paragraph 5) - The regulatory framework for NGT products is under development and it is pre-emptive to assume that detection will be part of the requirements, should such be mandated by future regulations. Moreover, as cited in section 1.2 and in previous EURL reports, methods for detection and quantification of such edited products are not yet well established or understood. Thus, MPR recommendations are premature for NGT’s.

Section 2.1.1 Acceptance criteria common to all modules of a method. Practicability – Many aspects of a digital PCR reaction are specific to the platform being used. It is not practicable nor feasible to directly transfer a dPCR method from one platform to another when specific platforms use proprietary reaction components, manufacturer-recommended primer/probe concentration, and specific reaction volumes which are all critical to the initial method validation. As is stated in the guidance, further optimisation may be required across platforms. What optimisations would be acceptable for a successful transfer or would the method be validated as solely dedicated to the instrument platform on which it was developed? Likewise, a method validated for dPCR, including the DNA extraction module, may not be transferable to real-time PCR format considering that dPCR is less sensitive to impurities from DNA extraction.

We suggest providing a minimum set of data requirements for the assay, which would then need to be further developed to be used on a separate platform.

2.1.3 Acceptance criteria applicable to digital PCR modules. Specificity – Separation of positive and negative clusters may be difficult, especially when applying validated qPCR methods to dPCR platforms. A duplex assay utilizing fluorophores with overlapping emission spectra (i.e., FAM and HEX) may show bleed-through from one channel to the other. This is a technical limitation of, for example, BioRad QX100/QX200 ddPCR which has only two fluor channels. Additionally, some digital instruments provide peak data in different formats. One could minimize bleed-through from one channel to the other, for example, by lowering primer/probe concentration, but this could lead to elevated intermediate fluorescence. There is a balance between two desired outcomes. Further, endogenous reference assays are validated at specific conditions. This limits flexibility to change and optimize assays for dPCR. There should

be practical considerations to allow for small changes to PCR parameters (excluding changes to primer and probe sequence) when transitioning between qPCR and dPCR, without the need for a full validation. We ask EURL-GMFF to confirm which small changes would be acceptable.



2.1.3 Acceptance criteria applicable to digital PCR modules. Specificity and Annex 1 Quality criteria for Reactions – Intermediate fluorescence signals (also known as “rain”) limited to 2.5%. However, not all dPCR manufacturer software provides data as utilized in the lambda calculations mentioned in Lievens et al. 2016. Please provide an example calculation that is applicable across multiple digital platforms.

2.1.3 Acceptance criteria applicable to digital PCR modules. Specificity – Please provide an explanation if there is an expectation that the cluster partitioning data needs to be shown in the validation report. In MPR part 2 Annex section Unit of Measurement, it is stated that the specificity may be measured by the separation of the positive and negative clusters following (Rs) measurements cited by Lievens et al., 2016. The measurements stated may not be feasible with some digital platforms as the data are not provided in the same format as the Bio-Rad droplet digital instrument. We suggest, as an alternative, to accept the graphical output from the digital software.

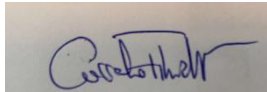
2.1.3 Acceptance criteria applicable to digital PCR modules. Dynamic Range – Original MPR requires dynamic range of the taxon-specific module be between 50- 56000 copies. 56000 copies may exceed the technical capability of some dPCR platforms making this requirement unachievable for a digital assay or may negatively affect other performance parameters due to an overload of DNA. Correspondingly, the lower limit of 50 copies quantification may be too low to accurately quantify. For example, while some manufacturers criteria claim sensitivity up to 100,000 copies, practical experience for some crops in the lab finds a maximum copy number of ~20,000 to be optimal and allow for compliance with specified parameters. In consideration of other criteria, we suggest that the dynamic range expectations be re-aligned.

2.1.3 Acceptance criteria applicable to digital PCR modules. Robustness – Testing alternate platforms is not practical nor completely feasible due to challenges in finding multiple external laboratories that provide this technology. Alternate dPCR platforms are 1. Vastly different technologies, 2. Use proprietary master mixes, and unique PCR conditions and 3. Do not consistently provide the same data output.

Changing temperature ramp rate is not possible on all dPCR platforms. The Qiagen QIAcuity and the ThermoFisher Absolute Q have pre-set ramp rates that are not adjustable. While the Bio-Rad ramp rate is adjustable, Bio-Rad recommends the ramp rate to be 2.5°C/sec. This allows the innermost droplets to reach an appropriate temperature. According to the manufacturer, increasing ramp rates could increase intermediate fluorescence signals (‘rain’). We suggest that these two requirements for robustness should be removed.

Annex 1. Recommendations for digital PCR methods. Method Optimization –In Lievens et al. 2016, the authors developed an R-script to calculate the rate of intermediate fluorescence specifically for droplet dPCR. The data may be provided in different formats across current platforms, including the current version/some versions of Bio-Rad ddPCR software (QX software), therefore, calculation using the R-script may not be possible. Please provide an example of this calculation that is applicable across multiple digital platforms.

Yours sincerely,

A rectangular image showing a handwritten signature in blue ink. The signature is cursive and appears to read 'Corrado Finardi'.

Corrado Finardi
Director Regulatory Affairs
CropLife Europe