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Research paper

Optimization of digital droplet polymerase chain reaction for quantification of genetically modified organisms

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ABSTRACT

Digital PCR in droplets (ddPCR) is an emerging method for more and more applications in DNA (and RNA) analysis. Special requirements when establishing ddPCR for analysis of genetically modified organisms (GMO) in a laboratory include the choice between validated official qPCR methods and the optimization of these assays for a ddPCR format. Differentiation between droplets with positive reaction and negative droplets, that is setting of an appropriate threshold, can be crucial for a correct measurement. This holds true in particular when independent transgene and plant-specific reference gene copy numbers have to be combined to determine the content of GM material in a sample. Droplets which show fluorescent units ranging between those of explicit positive and negative droplets are called 'rain'. Signals of such droplets can hinder analysis and the correct setting of a threshold. In this manuscript, a computer-based algorithm has been carefully designed to evaluate assay performance and facilitate objective criteria for assay optimization. Optimized assays in return minimize the impact of rain on ddPCR analysis.

We developed an Excel based 'experience matrix' that reflects the assay parameters of GMO ddPCR tests performed in our laboratory. Parameters considered include singleplex/duplex ddPCR, assay volume, thermal cycler, probe manufacturer, oligonucleotide concentration, annealing/elongation temperature, and a droplet separation evaluation. We additionally propose an objective droplet separation value which is based on both absolute fluorescence signal distance of positive and negative droplet populations and the variation within these droplet populations. The proposed performance classification in the experience matrix can be used for a rating of different assays for the same GMO target, thus enabling employment of the best suited assay parameters. Main optimization parameters include annealing/extension temperature and oligonucleotide concentrations.

The droplet separation value allows for easy and reproducible assay performance evaluation. The combination of separation value with the experience matrix simplifies the choice of adequate assay parameters for a given GMO event.

1. Introduction

Digital polymerase chain reaction (dPCR) is an emerging method for a growing number of applications [1]. In contrast to classical real-time PCR (qPCR) where amplification is performed in one single reaction volume (e.g., 25 μL), in dPCR the reaction mix is partitioned into thousands of tiny reaction cavities for individual PCR runs. By counting each cavity and detecting whether PCR amplification has taken place (positive) or not (negative), absolute copy numbers of target DNA can be calculated. Using thousands of droplets on a nanoliter (nL) scale is a flexible and relatively cost-efficient version of dPCR, called droplet digital PCR (ddPCR). One popular system for ddPCR is Bio-Rad's QX system [2].

Defining the fluorescence threshold that separates positive from negative reactions is not always straightforward. Droplets exhibiting fluorescence ranging between those of explicit positive and

Abbreviations: ABI, LifeTechnologies (formerly AppliedBiosystems); Bio, DNA Technology/Biosearch Technologies; Cat. No., catalogue number; cp/cp, (gene) copy per (gene) copy; dPCR, digital PCR; ddPCR, droplet digital PCR; DNA, deoxyribonucleic acid; EC, European Commission; ERM, Certified European Reference Material; EU, European Union; EURL-GMFF, European Reference Laboratory for GM Food and Feed; fluorescein, FAM,F; gDNA, genomic DNA; GM, genetically modified; GMO, genetically modified organism; HEX,H, hexachlorfluorescein; L, liter; Lec, lectin gene of soy; MeanSignal, mean fluorescence signal value; MIQE, minimal information for publication of quantitative digital PCR experiments; MRPL, minimum required performance limit; MS, Microsoft; MWG, Eurofins-MWG; qPCR, (quantitative) real-time PCR; PCR, polymerase chain reaction; SD, standard deviation (of fluorescence signals); TAMRA,T, tetramethylrhodamin; Tech, technician; TIB, TIB Molbiol; VBA, visual basic for applications; VIC,V, fluorescent dye (LifeTechnologies).

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negative droplets are called 'rain'. The origin of the rain is not clear. Rain often is attributed to delayed PCR onset [3] or partial PCR inhibition in individual droplets [4]. However, it could also be a consequence of damaged positive droplets with corresponding reduced fluorescence, or damaged negative droplets with increased background fluorescence, or a mixture of both [5].

The existence of rain can hinder analysis and the correct setting of a threshold. Several approaches exist to minimize the effects of rain on quantitative results [3,5]. Unfortunately, the existing algorithms like 'definetherain' [5] consider only the FAM channel of the QX ddPCR system, while disregarding the HEX/VIC channel.

An important task of official food and feed control in the European Union (EU) is to monitor the compliance of products with regulations related to labeling by appropriate quantitative laboratory analysis [6]. As the results of quantitative analysis can imply serious legal and financial consequences, especially in the light of Regulation (EU) No 619/2011 [7] for producers or distributors of feed, the quantification results need to be reliable. Tolerable traces of not-yet approved GMO in feed must not exceed the so-called 'minimum required performance limit' (MRPL), which is defined as corresponding to 0.1% mass fraction of genetically modified material [7].

It should be pointed out that to quantify GMO content in a sample at a level around 0.1% mass presents a special challenge as official PCR quantification methods usually have a validated dynamic range between 0.1 to 4.5% mass. This means that GMO falling under the scope of Regulation (EU) No 619/2011 [7] have to be quantified at the lower end of the dynamic range of these qPCR methods.

Almost all official quantitative detection methods published by the EURL-GMFF are so far based on qPCR with hydrolysis probes [8]. Several authors have however shown the potential of ddPCR for analysis of genetically modified organisms (GMO) [9–14]. Special requirements when establishing ddPCR for GMO in a laboratory include the choice between validated official qPCR methods and the optimization of these assays for a ddPCR format. Differentiation between droplets with positive reaction and negative droplets can be crucial for a correct measurement. This holds true in particular when independent transgene and plant-specific reference gene copy numbers have to be combined to determine the GM content of a sample [15]. After quantification of both the transgene and a species-specific reference gene, the corresponding mass fraction has to be calculated while considering the (assumed) zygosity of the plant tissue(s) and plant species under investigation [16].

Consideration of both FAM and HEX/VIC channels is therefore essential when transgene and reference gene are to be analyzed together in a duplex reaction. In this manuscript, a computer based algorithm has been carefully designed to minimize the impact of rain on ddPCR analysis, offering a more objective platform for assessment of ddPCR results. Our approach graphically visualizes the effects of experimental parameter variation on the quality of droplet separation. One application is a user-friendly quick overview of the already tested variations, in order to facilitate choice of the best assay parameters for a given analytical task.

2. Materials and methods

2.1. Samples

Certified reference materials of GMO events were either purchased from IRMM (Geel, Belgium), or from AOCS (Urbana, USA). Ground dry material was stored protected from humidity in a fridge at around 5 °C, DNA frozen at −20 °C. Multi-target plasmids for event maize NK603 were designed in-house and subsequently synthesized, propagated, purified and linearized by Eurofins-MWG (Ebersberg, Germany). Stock solutions of plasmids were kept at −80 °C, working solutions either frozen at −20 °C for long-term storage, or kept in the fridge at around 5 °C for usage within days.

2.2. DNA extraction

Genomic DNA (gDNA) was extracted from 100 mg (soy) or 200 mg (maize) ground dry material with the Maxwell 16 instrument (Promega, Mannheim, Germany) using a modified protocol [17]. Some batches of isolated gDNA were further purified with DNA Extractor Cleaning Columns Kit (Eurofins-GeneScan). Genomic DNA was not enzymatically digested prior to ddPCR if not otherwise indicated, plasmids were purchased linearized. Extracted DNA was either frozen at −20 °C for long-term storage, or kept in the fridge at around 5 °C for usage within days.

2.3. Oligonucleotides

Oligonucleotide primers and hydrolysis probes were synthesized by TIB Molbiol (Berlin, Germany), Eurofins-MWG, DNA Technology/Biosearch Technologies (Risskov, Denmark) or LifeTechnologies (formerly AppliedBiosystems, Carlsbad, USA) in HPLC-grade. Oligonucleotide sequences for the GM events in this study were obtained from the official EU method collection [8]. For references on oligonucleotides see Supplementary Tables 1 and 2.

Probes were labelled either with FAM (F in the matrix data), HEX (H), or VIC (V). The majority of probes were quenched with non-fluorescent black hole quenchers (without indication in the matrix data). Few probes were quenched with fluorescent TAMRA (indicated by an additional T in the matrix data).

2.4. ddPCR

Droplet digital PCR (ddPCR) was performed in investigator's laboratory with either a CFX96 or T100 PCR thermocycler with gradient function (both Bio-Rad, Munich, Germany). Samples were analyzed as technical duplicates. As master mix the 'ddPCR Supermix for Probes' (Cat. No. 186-3010, Bio-Rad) was used. The total reaction volume was either 20 μL or 22 μL, containing 1× master mix, primers and probes as stated above in section 'Oligonucleotides' and 5 μL of sample DNA, or water for negative controls. Oligonucleotide concentrations were as given in the method protocols ('normal'; Supplementary Tables 1 and 2 [8]) or—if otherwise indicated—900 nM for primers and 250 nM for probes ('high'). Oligonucleotide concentrations in the matrix are given as concentrations of primer 1, primer 2, and probe. 20 μL of the reaction mixture was then loaded on eight-channel disposable droplet generator cartridges (before 12.05.2014 Cat. No. 186-3008, from 12.05.2014 Cat. No. 186-4008, gaskets Cat. No. 186-3009, Bio-Rad). Droplets were generated with 70 μL of droplet generation oil (Cat. No. 186-3005, Bio-Rad) in the droplet generator of the QX100 system (Bio-Rad). The generated droplets were transferred to a 96-well PCR plate (Cat. No. 0030128.613, TwinTec, Eppendorf, Hamburg, Germany). The transfer was either done with a manual 1-channel 100-μL-pipette (Reference, Eppendorf) or with an automatic 8-channel 50-μL-pipette (Rainin E8-50XLS+, filter tips Cat. No. 17002927, Mettler-Toledo, Giessen, Germany).

After thermal sealing with pierceable foils in a PCR plate sealer PX1 (both Bio-Rad, foil Cat. No. 181-4040), the following temperature profile was used for PCR: 600 s 95 °C, and 45 cycles of 15 s 95 °C, and 60 s 60 °C. Temperature gradients —when indicated— on the thermocyclers CFX96 and T100 consisted of 61.0 °C, 60.7 °C, 60.0 °C, 58.8 °C, 57.4 °C, 56.2 °C, 55.4 °C, and 55.0 °C. After PCR the sealed plates were placed in the droplet reader from the QX100 system