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

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
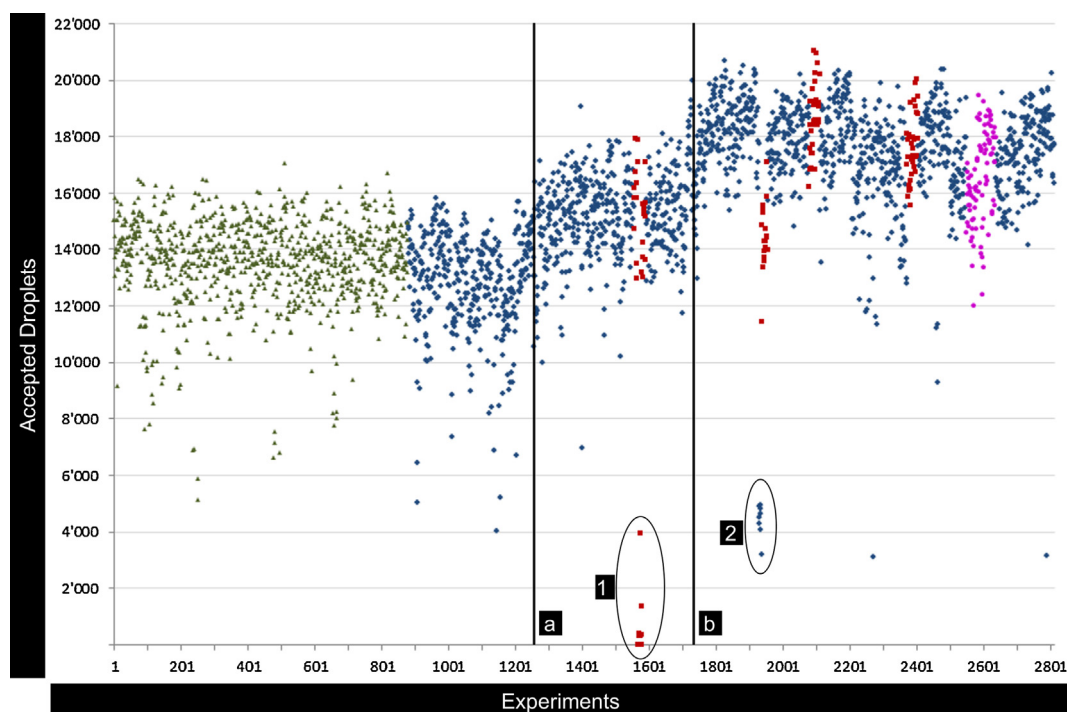
Fig. 1. Droplet recovery: The figure shows the numbers of accepted droplets (ordinate) for experiments on GMO analysis in our laboratory (abscissa; the numbers were given consecutively for all 2800 PCR reactions performed, duplex reactions were counted only once). Green triangles represent droplet populations transferred with a manual 1-channel pipette to the 96-well PCR plate, blue diamonds represent droplet populations transferred with an automatic 8-channel pipette; both were handled by the same technician (Tech1). Red and purple squares represent automatically transferred populations by two other technicians (Tech2, Tech3). (1) and (2) are outliers, while (a) and (b) represent shifts (see Section 3.1).

(Bio-Rad) and droplets were analyszed according to manufacturer's recommendations (Droplet Reader Oil Cat. No. 186-3004).

2.5. Data analysis

Droplet fluorescence data were initially analyzed with QuantaSoft software (Bio-Rad) versions 1.3.2.0 (from 25.09.2013) and 1.5.38.1118 (from 12.05.2014). Raw data, i.e., the fluorescence values for the droplets were exported from QuantaSoft software into Microsoft Excel 2010. Further analysis was done using the built-in functions and self-programmed VBA algorithms in the software. An Excel tool is available upon request from the corresponding author (or optionally available online). This Excel tool semi-automatically categorizes the fluorescence values of the droplets in positive and negative classes and the so-called 'rain' in-between [5]. Depending on the separation of positive and negative droplets (assay performance), an objective separation value k is calculated automatically. Together with other assay parameters (e.g., average copies per droplet for transgene and reference gene, number of accepted droplets, identified rain droplets) the determined separation value k can be semi-automatically exported from the original Excel tool and pasted into another Excel tool called the 'matrix' (see Supplementary 'Short manuals for accompanying Excel files').

2.6. Calculation of GMO percentages

Another feature of the first mentioned Excel tool is the possibility for the semi-automatic calculation of GMO percentage tables. This is achieved for duplex assays (transgene and reference gene) using Excel's built-in Table function. GMO percentages for different threshold combinations of transgene and reference gene assays can be automatically computed. Thresholds were considered separately, while resulting GMO percentages are given at the corresponding intersections in the table.

3. Results

3.1. Droplet recovery

Droplet digital PCR presented here is based on distributing the reaction mix into a multitude of partitions (theoretically up to 20,000). We documented the number of analyzable partitions (represented by the 'Accepted Droplets' in the software QuantaSoft) together with set-up-specific parameters for each run in our laboratory (Fig. 1). We have analyzed more than 2,800 droplet populations so far. A droplet population results from 20 μL of master mix including sample DNA, i.e., the read-out of a single well of the PCR plate.

The amount of accepted droplets could be raised by replacing a manual single-channel pipette by an automatic 8-channel pipette and additionally optimizing the pipette handling procedure by the technicians Fig. 1.

Additionally, the transfer of the procedure to other laboratory technicians was straightforward with the automatic model, resulting in comparable amounts of droplets. Outliers with few accepted droplets (<6,000) still occurred, but infrequently (Fig. 1, subpopulation 1 and 2).

An additional effect on the droplet distribution was observed when new cartridges (186–4008) for droplet generation and a new QuantaSoft version were used. The according shift of around 1,500 more droplets per population is detectable (Fig. 1, shift a). This shift coincides with the introduction of a new QuantaSoft version (1.5.38.1118) in our lab. A second shift was observed as a result of training and optimization effects (Fig. 1, shift b).

3.2. Threshold setting

The droplet reader used (QX100 system) is able to discriminate between signals from two channels: FAM and HEX/VIC. In our experience droplet separation in the FAM channel was noticeably better
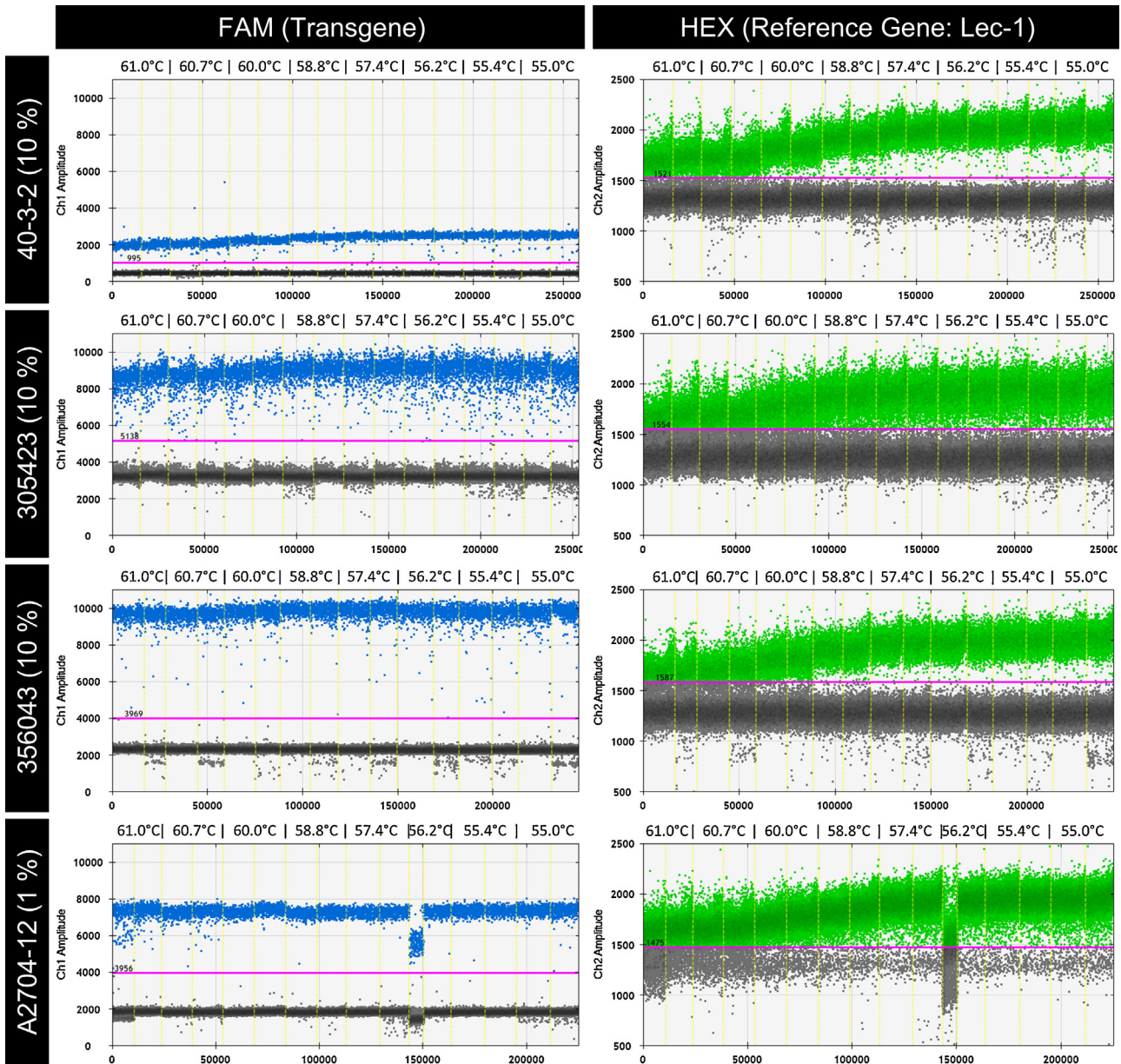
Fig. 2. Temperature gradients for four GM soy assays – Droplet view: The figure shows the droplet populations for single assays run in duplex: fluorescence amplitude (ordinate) for each droplet (abscissa). The names and percentage of the soy events measured are given on the left. Blue and green dots represent the positive droplets (above the pink horizontal threshold) for transgene and reference gene, respectively. Grey dots represent the negative droplets. A temperature gradient was applied for both the transgene and reference gene assays.

than in the HEX/VIC channel. Although this is not always the case, the possibility that this phenomenon occurs is greater when the positive droplets are much more abundant against the backdrop of negative droplets in that channel. Therefore, in GMO duplex analysis the FAM channel was used for transgene detection which is usually less present than the plant specific reference gene (Fig. 2).

We exemplarily analyzed the difficulties in setting a correct threshold to separate the droplet populations by calculating the corresponding GMO content of a well-characterized reference material (Fig. 3). Using 25 different thresholds for the transgene and 15 different thresholds for the reference gene, we determined the resulting spread in calculated GMO contents (Fig. 4). When the separation is good (57.4 °C in Fig. 3) the variation of the results (GMO% (cp/cp))—differing by a maximum of 2% from the result obtained by using pre-defined hypothetical ideal thresholds for FAM and

HEX/VIC channel (this GM content is given in the upper left corner of the tables from Fig. 4)—allows for many different positions of the thresholds to give similar GMO contents (green region in Fig. 4). Worse separation in the reference gene, i.e., here observed at higher annealing temperatures, narrows down the region with a maximum of 2% difference for the corresponding HEX threshold (60.0 °C and 60.7 °C in Fig. 4).

Another effect of the separation and thus the assay performance is the influence on the absolute calculated GMO content. The measured reference material (Fig. 3) had a nominal GMO content of 10% for soy event 356043. The better the distinction between positive and negative droplets, the smaller the effect of the positioning of the threshold, and, even more importantly, the closer the measured GMO content resembled the nominal value (Fig. 4).
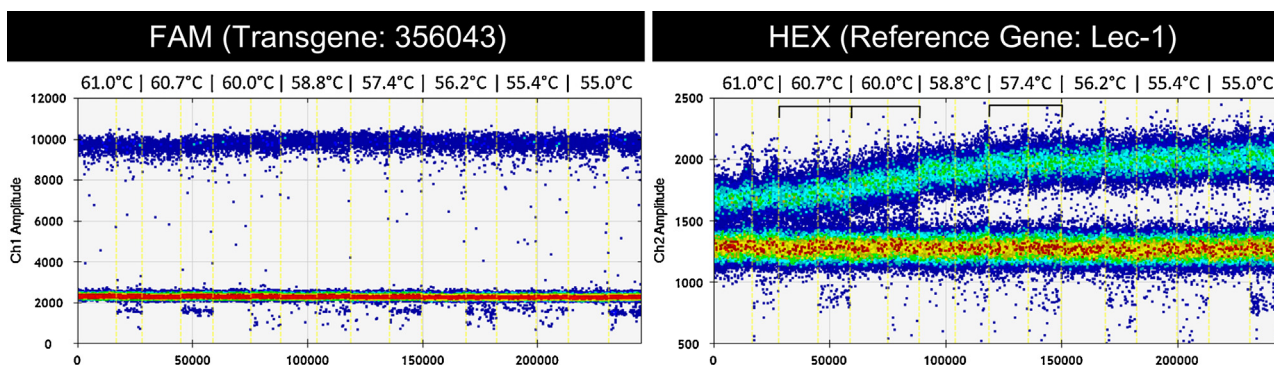
Fig. 3. Discrimination between positive and negative droplets: Measurement of soy event 356043 1% ERM (BF425c). The figure shows the droplet populations for single assays run in duplex: fluorescence amplitude (ordinate) for each droplet (abscissa). False colours represent the droplet concentration (blue for low and red for high concentrations). A temperature gradient from was applied for both the transgene and reference gene assays.

3.3. Temperature gradients and oligonucleotide concentrations

One recommended way for improving separation between positive and negative droplets in ddPCR is lowering the annealing/extension temperature of the PCR [18]. Ideally, this is tested with a thermal cycler that offers a temperature gradient function. By using such a cycler, the influence of up to eight different annealing/extension temperatures could be compared in a single PCR run.

The effect of the annealing temperature on separation between positive and negative droplets was already visible for the reference gene in the threshold setting example (Figs. 3 and 4). The improvement of droplet separation with reducing annealing/extension temperature was most prominent in the HEX/VIC channel but could sometimes also be observed in the FAM channel (data not shown). As the separation is generally good for the FAM-labelled assays, the consequences for HEX/VIC-labelled assays are more interesting. Here, the extent of the improvement in droplet separation varies even for the same reference gene, depending on the partner assay (transgene assay) in the duplex ddPCR (Fig. 2).

The identification—and especially the subsequent quantification—of GMO plants is based in the EU mainly on real-time PCR methods validated and published by the EURL-GMFF [8]. When starting with ddPCR, we tried to stick as close as possible to the protocols of these official methods, encouraged by a report showing the applicability of these methods even in duplex reactions without further modification [12]. Consequently, we kept the oligonucleotide concentrations as published, and changed merely to HEX labelled probes for reference gene assays and to black-hole quenchers where applicable for transgene and reference gene assays.

The manufacturer's manual for the ddPCR master mix [19] recommends high oligonucleotide concentrations of 900 nM for primers and 250 nM for probes which are unusual for real-time PCR in the case of GMO quantification. We tried these higher concentrations in combination with the already described temperature gradients (Fig. 5). The higher oligonucleotide concentrations resulted in raised signals both for the positive and the negative droplets. Nevertheless, depending on the assay, the threshold setting must be given careful consideration.

Additionally, there are hints for performance differences when comparing probes from different suppliers (data not shown).

3.4. Matrix and assay selection

In the course of establishing ddPCR for GMO analysis in our laboratory we varied several reaction parameters, such as annealing/elongation temperatures, oligonucleotide concentra-

tions, thermal cyclers and probe manufacturers. So far, we applied a total of 24 different assays for transgene detection (Supplementary Table 1) and 7 reference gene assays (Supplementary Table 2). For better comparison of ddPCR performance by means of droplet separation derived from different experimental parameters, we deposited our findings in an Excel Table resulting in a data matrix with currently 309 datasets.

One of the most important empirical findings is the performance of the assay, expressed in the proposed continuous separation value k (see below). The separation value k incorporates the objective parameters of background fluorescence of negative droplets versus fluorescence signal from positive droplets. This is combined in the matrix with the more subjective discrete parameter of a separation rating by means of ease to set an appropriate threshold.

Our matrix can be analyzed via the Pivot functions of Excel, resulting in Pivot charts illustrating both objective background/signal values and subjective separation ratings in a graphical way. The Pivot charts allow for presentation of condensed information from the droplet clouds, e.g., for temperature gradients from Fig. 2 in the Pivot equivalent in Fig. 6, or for additional different oligonucleotide concentrations (both panels in Fig. 5).

With the aid of the matrix/Pivot charts, the information for certain assays can be quickly and easily accessed, without lengthy search in copious tables. Exemplary overviews for soy 40-3-2 transgene and reference gene assays are depicted in Fig. 7. Starting from an overview, suitable assay parameters can be visually identified, or, the other way round, unsuitable parameters excluded. In this example and from the parameters tested so far, best conditions for the assay would be as follows: Primers and probe according to Kuribara et al. [20], duplex PCR, high primer and probe concentrations (Fig. 7).

3.5. Objective separation value for classification of assay performance

Initially we manually selected a separation category (none, moderate, good or very good separation) for each assay, mainly based on the separation between positive and negative droplets, including the amount of signals in-between—the so-called 'rain'.

As this was a subjective measure prone to differences in analyzing from person to person (even the same person is likely to judge the same assay differently on another day), we strived for an objective way to classify assay performance. Based on the definition for rain from the 'definethe rain' algorithm [5], we developed a measure for separation between positive and negative signals (droplets) by
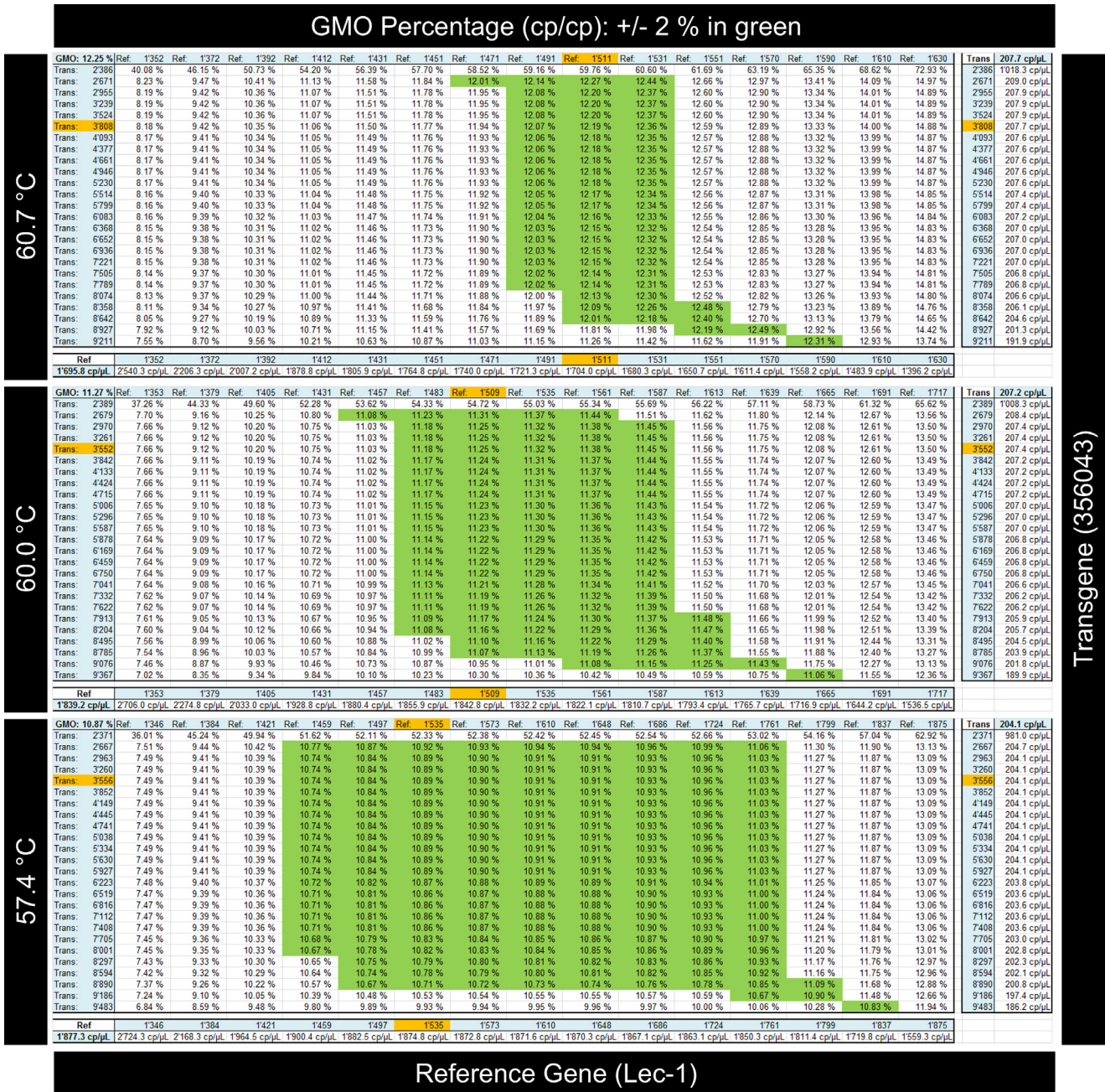
GMO Percentage (cp/cp): +/- 2 % in green

[Figure 4: Large data table showing GMO percentage measurements at three annealing temperatures — 60.7 °C, 60.0 °C, and 57.4 °C — with transgene (356043) thresholds on the ordinate and reference gene (Lec-1) thresholds on the abscissa.]

Fig. 4. Detailed effects of threshold setting and annealing temperature on measured GMO content: Results for measurement of soy event 356043 10% ERM (BF425c) for three annealing temperatures (Figure 3). Thresholds for transgene and reference gene are given on the ordinate or abscissa, respectively. GMO percentages deviating by a maximum of 2% from manually pre-defined thresholds (coloured in orange), are marked in green. The underlying target gene concentrations are given for comparison of the border regions.

the determination of the separation factor k that would solve the following equation:

$$\text{MeanSignal}_{pos} - k \times \text{SD}_{pos} = \text{MeanSignal}_{neg} + k \times \text{SD}_{neg}, \text{ that is}$$

$$k = \frac{\text{Mean}_{pos} - \text{Mean}_{neg}}{\text{SD}_{pos} + \text{SD}_{neg}}$$

The factor k was calculated automatically using Excel's built-in goal-seek function. In this equation, k is the separation value, MeanSignal is the mean fluorescence signal of the positive or negative droplet population, and SD is the standard deviation of the positive or negative droplet population's fluorescence signals.

In conclusion, the higher the value of k, the better the separation of positive and negative signals (droplet populations).

By comparison of the calculated values for the assays with the manual categorization, the classes could be objectively defined by the borders given in Supplementary Table 3 for FAM-labelled probes, or Supplementary Table 4 for HEX/VIC-labelled probes, respectively. Different classification values for FAM and HEX/VIC probes were selected because of the generally better separation observed in the FAM channel, compared to the HEX channel.

The determined classification factor is influenced by the abundance of positive droplets but is in principle quite robust in reproducibly determining a category (Supplementary Fig. 1).
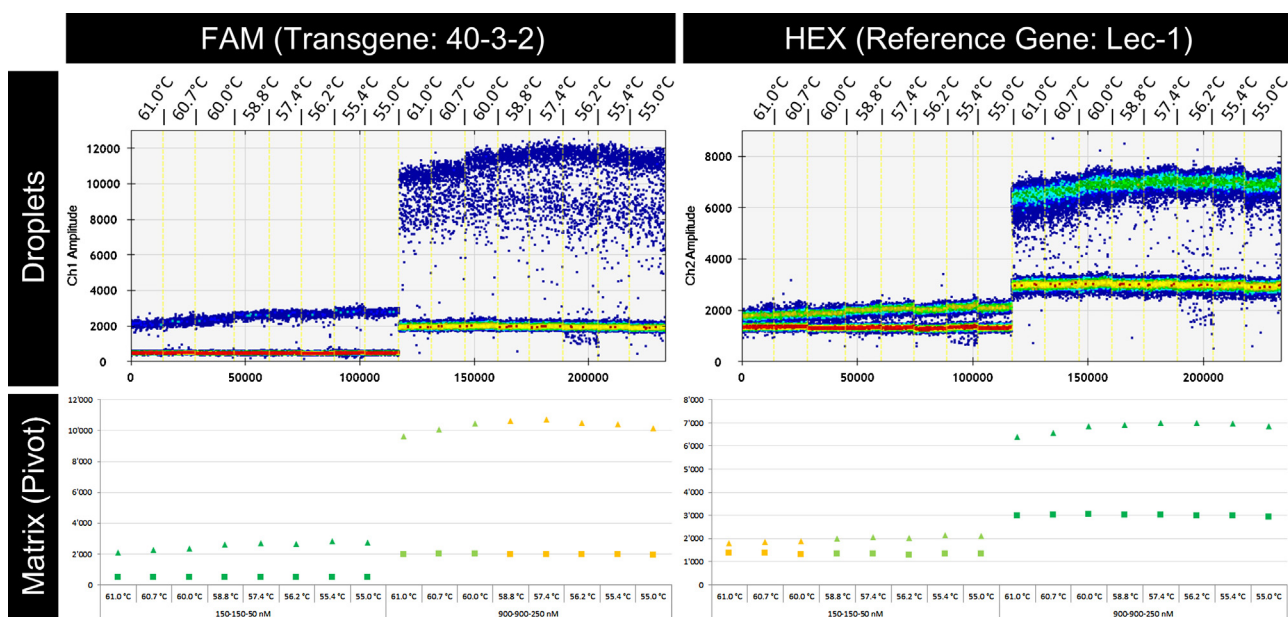
Fig. 5. Effect of annealing temperature and oligonucleotide concentration: The upper part of the figure shows the droplet populations for single assays run in duplex. The PCR setup was done with two different oligonucleotide concentrations. A temperature gradient was applied for both the transgene and reference gene assays with both oligonucleotide concentrations. The lower part of the figure shows the corresponding condensed information as Pivot charts from the matrix. For symbols refer to Fig. 6.

Our Excel tool can analyze the data by either removing the rain droplets from further calculation, or by using a manually set threshold for calculation of copies per μL and GMO contents. However, the main purpose of the developed Excel tool is not the removal of rain droplets from further analysis [done as in 5] but rather identifying assays with good separation according to the factor k. The larger the separation factor, the better the separation and the easier the selection of an appropriate threshold.

The determined separation value k is combined with other assay parameters into a single dataset. This dataset can be semi-automatically exported from the original Excel tool and pasted into another Excel tool called the 'matrix'.

The matrix contains information about all performed assays (Supplementary Tables 1 and 2) including the separation values. The separation values can be classified semi-automatically into the four separation categories: none, moderate, good and very good (Supplementary Tables 3 and 4). The matrix data can be visualised using Excel's built-in Pivot diagram features. The separation categories are coded by different colours (e.g., Fig. 6). The Excel matrix with the Pivot visualization is available upon request (or optionally available online).

3.6. Key features of the developed Excel tool

We propose a workflow starting from a run file in QuantaSoft and ending in a performance factor k used for classification in the Pivot matrix (for details see Supplement: Short manuals for accompanying Excel files).

The Excel tool for import, export and data analysis can automatically import raw fluorescence data from files in a given folder. After selection of the correct assay type (singleplex/duplex and used fluorescence channel), droplets are clustered iteratively based on their fluorescence signal into positive, negative and rain. The resulting copies per μL and GMO content are displayed. Based on the clustering, the performance factor k is also automatically calculated. All calculated assay parameters including k can be automatically exported as one dataset for later transfer to the experience matrix.

This Excel tool can be used for additional calculations. The threshold setting for FAM and HEX/VIC channel can be separately switched from the 'definetherain' algorithm to manually set threshold, with immediate display of result on copies per μL and GMO content.

The most powerful function of the Excel tool is the possibility to automatically generate data tables with GMO contents for 375 different combinations of FAM and HEX/VIC threshold values. One use of such Table is to directly study the magnitude of the effect caused by threshold setting. Another application is the empirical search for the best threshold analyzing a reference material with known GMO content and unknown samples in a run.

4. Discussion

The introduction of Regulation (EC) No 619/2011 [7] has posed an additional challenge to GMO testing laboratories in the EU as quantification in the range of 0.1% has to be accurately achieved. Digital droplet PCR with its high sensitivity and independence from standard curves (measuring absolute DNA copy numbers) is therefore a very promising tool for GMO analysis (and other applications) especially at low DNA concentrations. As a valuable and powerful tool for quantitative GMO (DNA) analysis, it remains to be shown that ddPCR is really up to the task compared to qPCR. Several authors have shown that ddPCR can be used for quantification of certain GMOs [e.g.,12–14,21]. Our work intends to contribute to a better understanding of the dynamics of ddPCR in GMO analysis of food and feed, offering a more objective platform for sample evaluation.

4.1. Droplet recovery

Sensitivity is directly dependent on the number of analyzable partitions: the more partitions, the better the maximal achievable sensitivity. When using a chamber based system with a fixed number of reaction cavities, the sensitivity is fixed, provided that all cavities are equally filled. In ddPCR, the master mix is distributed into a variable number of droplets [22]. The partition number is also an essential criterion of the Digital MIQE Guidelines [1] as it is directly linked to sensitivity.
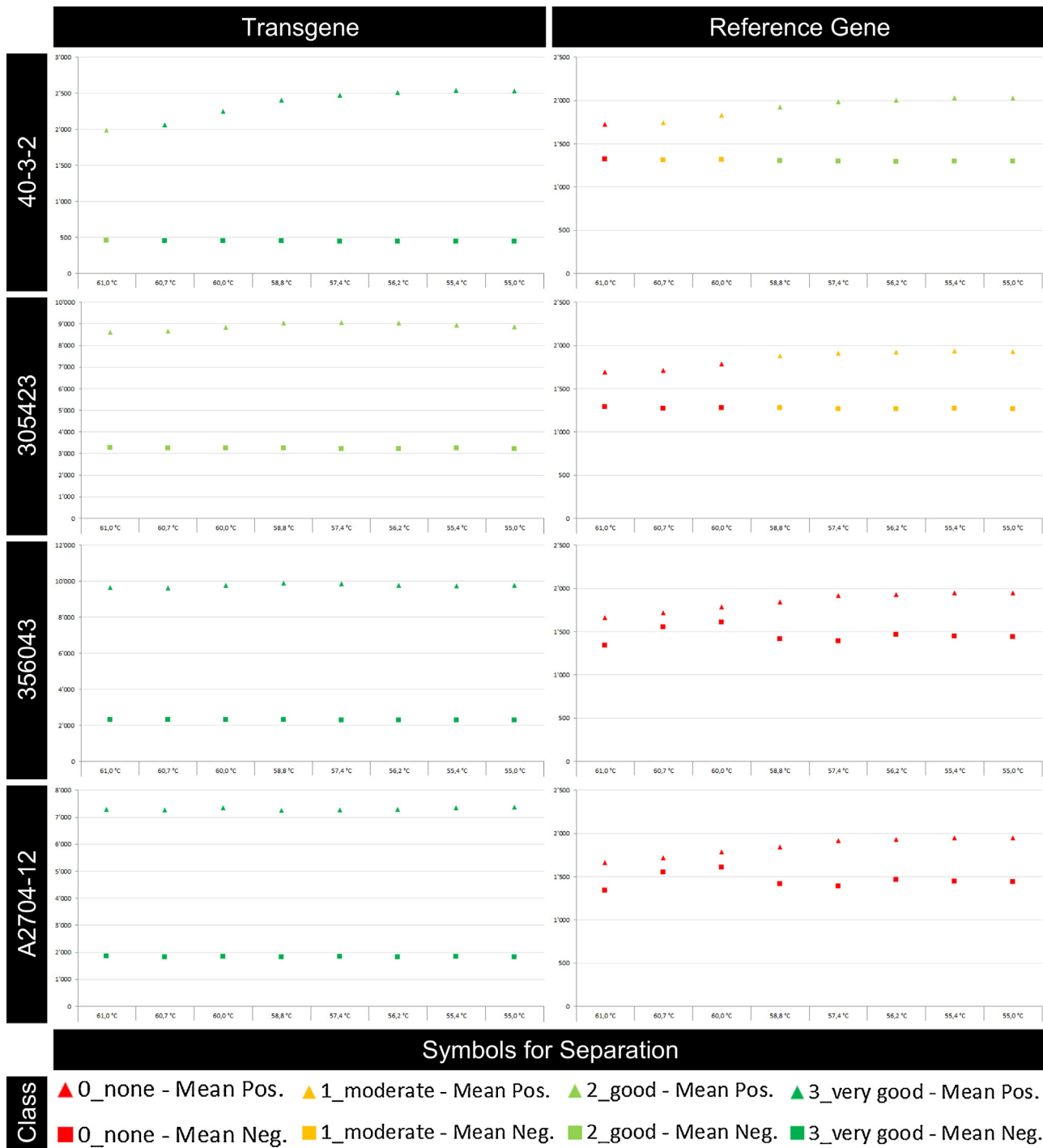
Fig. 6. Temperature gradients for four GM soy assays – Matrix view: The figure shows Pivot charts from our matrix. Coloured squares represent the approximate background and coloured triangles the approximate signal for the chosen Pivot options. Red, orange, light green and dark green represent none, moderate, good and very good separation, respectively.

One of our goals when starting with ddPCR in our lab was therefore to establish a sufficient and stable number of analyzable droplets. This droplet recovery is represented by the number of software-accepted droplets per generated droplet population, i.e., the read-out of a single well of the PCR microtiter plate. The number of accepted droplets generated in our lab (around 16.000 in average) was in good accordance with or even considerably higher than values for other published GMO analysis [12,21].

In our experience, a crucial manual step in ddPCR is the transfer of the fragile freshly generated droplets into the wells of the PCR plate with a pipette. This is due to the fact that transferring the mix with droplets with a constant low pipetting speed (suction) and an appropriate (steep) angle of filter tips touching gently the wall of the microtiter plates helps to minimize the mechanical disruption of droplets. The transition towards higher accepted droplet numbers (>16,000) after an initial training period is also reflected in Fig. 1 (shift b). We nevertheless recommend using an automatic 8-channel pipette for minimising variations between different operators. The cost for the automatic pipette model is low compared to other ddPCR consumables.
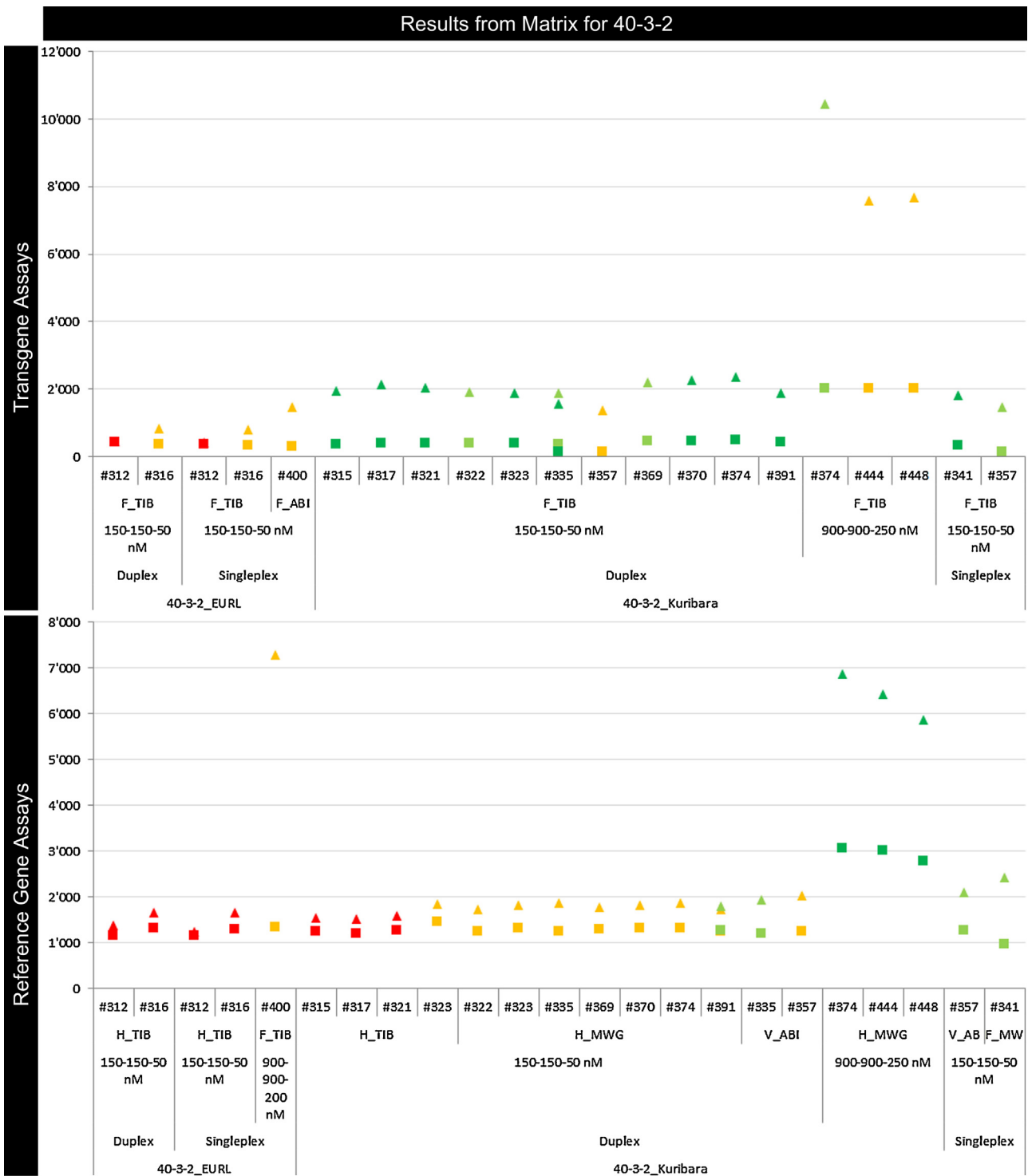
Fig. 7. Matrix overview for soy 40-3-2 event and reference gene assays: The figure shows Pivot charts from the matrix for the detection of soy event 40-3-2 under various conditions (single and duplex PCR, normal and high primer and probe concentrations, probes from different suppliers, all at 60 °C annealing/extension temperature). For symbols refer to Fig. 6.

According to Bio-Rad (personal communication) an additional increase of generated droplets results from the fact that droplets generated with the 186–4008 cartridges are consequently smaller (0.85 nL) compared to the droplets obtained with the 186–3008 cartridges, when the same 20 µL volume is used for droplet generation. Nevertheless this information clearly contradicts the findings of Corbisier et al. [23] and Dong et al. [24] who measured a constant droplet size of 0.83–0.85 nL generated with the previous cartridges (186–3008). According to these findings the increased number of droplets with the 186–4008 cartridges is caused by the fact that these cartridges are more efficient and transform a larger amount of the 20 µL sample into droplets. In addition, the update of the software on the droplet reader allows the droplet reader to pick up a larger volume from the PCR plate and therefore also more droplets.

4.2. Threshold setting

Differentiation between droplets with successful PCR amplification (positive droplets) and droplets without amplification (negative droplets) is the basis for the subsequent calculation of absolute copy numbers. A threshold, which can be set either manually or automatically by the software, usually separates positive from negative droplets. In order to retain as much control as possible over the distinction between positive and negative signals (droplets) we favored manual over automatic threshold setting. When using automatic threshold setting it is highly recommended to double check results obtained.

Some droplets however exist in the in-between and are neither clear positives nor negatives. These are usually called rain. As this rain can significantly alter the calculated copy numbers, attempts have been made to virtually eliminate the existing rain droplets from the calculation [3,5].

Before mathematically excluding rain from the calculation, we believe that a more appropriate approach would be an optimized assay, where the setting of the threshold should have little consequences on the calculated copy number.

The correlation between appropriate threshold setting and trueness in ddPCR analysis, although quite logical, has to the best of our knowledge, not been previously demonstrated. With our developed Excel tool, this could be demonstrated for ddPCR with transgene and reference gene assays in a duplex reaction (Figs. 3 and 4). Relying on the built-in Excel algorithms and VBA programming, a data pool of raw ddPCR results, could be semi-automatically imported into Excel. The effect of rain and adjusted threshold settings on analysis of GMO events could thus be comprehensively evaluated.

4.3. Temperature gradients and oligonucleotide concentrations

The methods for GMO analysis published by the EURL-GMFF [8] are based on qPCR with singleplex assays for transgene and reference gene, usually run at 60 °C annealing/elongation temperature with defined oligonucleotide concentrations. Using these published methods for ddPCR, e.g., for ddPCR with duplex assays for transgene and reference gene, is a significant deviation from the published and validated methods. Nevertheless such deviations have merit when thereby better quantification of GMO contents is achieved.

To improve the separation between positive and negative droplets, we lowered the annealing/extension temperature and raised the oligonucleotide concentration (Fig. 5). Both procedures enhanced the separation in many—but not all—assays tested. We propose to use the assay protocol with the best separation that remains as close as possible to the validated PCR method. Nevertheless, the assays with the determined optimal reaction parameters may eventually have to be validated or verified. Such verification could include the determination of parameters such as precision, trueness and accuracy [25].

For certain assays and matrices, an increase in cycle number might also be advantageous [26], however, we have not empirically tackled this possibility in our lab so far.

Whether and to which extent the supplier of the probes and/or each batch of the production has influence on the separation of positive and negative signals is yet unclear. We saw first hints for performance differences using probes from three different suppliers. Whether this was due to different internal quality controls, or may even be dependent on the produced lot, remains unclear and an open question for future testing.

4.4. Matrix and assay selection

As the fluorescence values for negative and positive droplets are important measures for each ddPCR assay, the Digital MIQE Guidelines [1] state that examples of end-point fluorescence values or graphic readouts should be included in manuscripts or supplementary material.

We expand on this requirement and suggest a matrix that combines these fluorescence values with an objective separation value for each tested assay (Fig. 7). The condensed information can then be the starting point for narrowing down to specific settings, or for identification of optimization needs.

In the depicted example of assays for 40-3-2 soy (Fig. 7), the methods of the EURL-GMFF—used in duplex PCR instead of singleplex PCR—are not suitable (shown on the left, runs 312 and 316) as they have only red or orange symbols, depicting none or moderate separation, respectively. The same holds true for methods of the EURL-GMFF in singleplex assays for event 40-3-2 and lectin, respectively. The 40-3-2 method according to Kuribara [20] used as singleplex assays would yield sufficient separation (shown on the right, runs 357 and 341 for lectin), with the cost of more—error-prone—pipetting steps and the need for additional sample DNA. Increasing the oligonucleotide concentrations did however significantly improve separation, even in duplex assays (run 374). When the annealing/elongation temperatures are displayed (not shown in the figure), it is clearly visible that a decrease in temperature would improve the separation at normal oligonucleotide concentrations (runs 370, 374 and 391). In general, for detection of soy event 40-3-2, it is necessary to deviate from the validated qPCR protocols in one way or another, to achieve a sufficient separation of positive and negative signals (droplets). In this case, the higher oligonucleotide concentrations with the common annealing/elongation temperature of 60 °C would be the preferred assay parameters.

4.5. Objective separation value for classification of assay performance

The existing and published algorithms for definition of rain are so far limited to the FAM channel [3,5]. We expanded this concept to the HEX/VIC channel and added an objective separation value. This novel objective separation value gives additional (and colour-coded) information for each assay on top of the fluorescence values for negative and positive droplets (Fig. 6).

The proposed separation factor k cannot be calculated directly from the data in QuantaSoft, as the SD needed for the positive and negative populations is not available. In consequence, a tool that gives these SD values has to be used. This could either be our Excel spreadsheet, or another tool that is able to cluster the fluorescence values of the droplet populations, e.g., the 'definetherain' algorithm [5], or another statistics package that is capable of analyzing datasets with thousands of fluorescence unit values. Unfortunately, the 'definetherain' algorithm is not designed to process data from the HEX/VIC channel.

Our developed Excel spreadsheet supports (raw) data from the QX system, both FAM and HEX/VIC channel. The separation factor k takes into account both the absolute fluorescence difference of negative and positive droplets, as well as the scatter of negative and positive droplet populations, respectively. It therefore combines both assay quality criteria: fluorescence difference and variation in the droplet populations.

The better the separation, the wider the range for correct threshold setting (Fig. 4). This is also reflected in the objective separation factor k. For the three representative temperatures 60.7 °C, 60.0 °C, and 57.4 °C the corresponding separations factors k for the reference gene (Lec-1) are 2.7, 3.2 and 4.3, for the transgene (356043)

18.4, 17.6 and 16.8, respectively. Separation for the transgene in the FAM channel is at all temperatures very good, which is visible in the broad vertical green range of 2% difference (Fig. 4). In contrast, separation in the HEX/VIC channel is weak (reflected by separation ratings none to moderate in the matrix, Fig. 6), visible in the considerably narrower horizontal green range of 2% difference. With increasing separation factor *k* the corresponding green range widens (Fig. 4, from top to bottom).

Cooperation with other labs to generate more datasets would be appreciated. Researchers applying the developed spreadsheet can categorize their own data in order to get a good impression about the effects of different settings on their assays.

We envision the presented approach to be used by researchers to investigate how the effects of different variables impact on performance in their laboratories. By pooling datasets from several laboratories, valuable conclusions could be drawn on reproducibility and repeatability of ddPCR. All generated datasets could subsequently be collected in a centralized and publicly available database or archive.

4.6. Key features of the developed Excel tool

The Excel tool could be upgraded to support further requirements in ddPCR analysis. One possibility would be to integrate direct analysis of samples with optimized thresholds generated for a reference material, similar to the 'definetherain' algorithm [5], but for immediate calculation of GMO contents using information from both FAM and HEX/VIC channels.

Excel and VBA programming might not be the best approach for future developments, instead, using a dedicated programming environment (like for example R or C#) may be better suited for implementation of the presented algorithms. The authors are open to suggestions for cooperation to implement such a transformation.

5. Conclusions

We developed an Excel based 'experience matrix' that reflects the assay parameters of GMO ddPCR tests performed in our laboratory. We therefore 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 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.

For transferring existing real-time PCR assays to a ddPCR platform, we recommend testing several reaction parameters and analyzing these with our developed experience matrix. Such parameters would include annealing/extension temperature and oligonucleotide concentrations.

Conflict of interest

The authors declare that there are no conflicts of interest.

Authors' contributions

LG designed the experimental setup, performed the data analysis, developed the corresponding MS Excel spreadsheets and drafted the manuscript. AI added experimental data, analyzed data and helped to draft the manuscript. UB provided instrumentation and helped to draft the manuscript. SP participated in the design of the study, provided reference materials, analyzed data and helped

to draft the manuscript. All authors read and approved the final manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bdq.2015.12.003.

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