Chapter 24

Droplet Digital PCR for Absolute Quantification of Pathogens

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Abstract

The recent advent of different digital PCR (dPCR) platforms is enabling the expansion of this technology for research and diagnostic applications worldwide. The main principle of dPCR, as in other PCR-based methods including quantitative PCR (qPCR), is the specific amplification of a nucleic acid target. The distinctive feature of dPCR is the separation of the reaction mixture into thousands to millions of partitions which is followed by a real time or end point detection of the amplification. The distribution of target sequences into partitions is described by the Poisson distribution, thus allowing accurate and absolute quantification of the target from the ratio of positive against all partitions at the end of the reaction. This omits the need to use reference materials with known target concentrations and increases the accuracy of quantification at low target concentrations compared to qPCR. dPCR has also shown higher resilience to inhibitors in a number of different types of samples. In this chapter we describe the droplet digital PCR (ddPCR) workflow for the detection and quantification of pathogens using the droplet digital Bio-Rad platform QX100. We present as an example the quantification of the quarantine plant pathogenic bacterium, *Erwinia amylovora*.

Key words Digital PCR, Droplet digital PCR, Plant pathogens, Absolute quantification, Viruses, Bacteria

1 Introduction

During the past decade real-time quantitative PCR (qPCR) has been progressively accepted as one of the golden standards for molecular detection and quantification of pathogens [1, 2]. The advantages of qPCR that have contributed to its wide use include reduced possibility of contamination by eliminating the need for post-reaction processing, improved multiplexing and throughput, real-time monitoring, and significantly its potential for quantification [3]. However, for quantification in qPCR a standard curve with known concentrations of the target is necessary to transform the output values of qPCR, quantification cycles (Cq), into actual concentration values (target copies/μl). The unavailability of standardized reference materials in plant pathology can lead to a

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lack of harmonization and significant inter-laboratory quantification biases. Moreover, the co-extraction of substances that influence the amplification efficiency (inhibitors and facilitators) influences the Cq value obtained in qPCR which can lead to lower accuracy of quantification [4]. qPCR also shows limitations when rare alleles or mutants are to be quantified or detected in a high background of wild type targets [5].

Digital PCR (dPCR), which originated from the studies of target quantification using limiting dilutions [6], has the potential to improve the above mentioned limitations of qPCR. In the dPCR format, the reaction mixture containing primer/probes and nucleic acid sample is divided into hundreds (BioMark™ HD System Fluidigm) to millions (RainDrop® System RainDance) of partitions (also called compartments or droplets). Depending on the dPCR platform, these partitions are constituted by chambers or droplets. A fraction of the partitions is occupied by a single to few copies of the target DNA/RNA, while a fraction of compartments must remain target free. After amplification, positive and negative compartments are counted and the absolute concentration of target copies in the initial sample is derived by applying Poisson's distribution without the need for any standard [7]. Compartmentalization facilitates the detection and quantification of rare mutants in a background of wild type sequences [8], because, in contrast to qPCR, low frequency targets do not need to compete for reaction mixture resources (primers, probes, …) with those present in high frequency leading to a higher signal to noise ratio. In dPCR, the number of positives and negatives is counted after end point PCR amplification, and therefore, the final result is independent of variations in the PCR amplification efficiency, making dPCR potentially more accurate, repeatable, and less prone to inter-laboratory variations than qPCR [9]. Several recent studies also suggest that dPCR can be more resilient to inhibitors than its non-digital counterpart [10–12].

Different dPCR platforms are available, which differ mainly in the strategy they use to produce partitions (chambers or droplets) and in their number [13]. Among the first instruments available, Fluidigm Corporation (San Francisco, CA) and Life Technologies (Carlsbad, CA) offer microfluidic chip based compartmentalization resulting in hundreds to thousands of compartments, while Bio-Rad (Hercules, CA) and RainDance (Lexington, MA) focus on emulsion based compartmentalization that can generate from tens of thousands (QX100 and 200 systems, Bio-Rad) to up to millions of droplets (RainDrop® System, RainDance). New platforms are also starting to emerge as well as upgrades of existing ones. The number of partitions affects the accuracy of quantification. This is of importance primarily in for example rare event detection in a high background. For the majority of applications

involving quantification of plant pathogens, the number of partitions in the range of 12,000–20,000 is sufficient.

Plant pathology field can benefit from dPCR features both for research and diagnostic applications. dPCR can enable accurate quantification of plant pathogens without standards, and is thus a promising method of choice for calibration of reference materials to be used in laboratories worldwide. dPCR can be applied to detect and study the dynamics of low frequent mutants within a given pathogen population, i.e., in quasispecies studies. Moreover dPCR can allow overcoming problems associated with the presence of inhibitors associated with certain plant materials and other difficult matrices (e.g., soil). Studies describing the application of dPCR to the quantification of pathogens are increasing [10, 14–16], among them also first examples dealing with plant pathogens, such as phytoplasma [17], *Erwinia amylovora* and *Ralstonia solanacearum* [18]. In our laboratory, pathogen quantification was successfully undertaken using droplet digital PCR (ddPCR). We found that optimized qPCR assays could be easily transferred to ddPCR format. In these assays, ddPCR offered higher quantification accuracy at lower concentrations [10, 17, 18] and lower sensitivity to inhibitors [10]. It has also been demonstrated that RNA quantification is possible using a one-step reverse transcription-ddPCR (RT-ddPCR) [10].

In summary, dPCR in general and in particular ddPCR have great potential for practical use in plant pathogen detection: (1) for quality control of in-house reference materials to be used in qPCR and other PCR-based diagnosis methods, (2) for quality control of materials used in test performance studies, (3) in optimizing and assessing qPCR and DNA/RNA extraction methods [18], and finally (4) for routine quantification of nucleic acid target in a wide variety of samples. While running costs of ddPCR currently remain slightly above qPCR, this is compensated by an improved performance (i.e., lower susceptibility to inhibition), ease of interpretation, and absolute quantification independent of reference materials. Among other advantages, multiplexing potential has been demonstrated for accurate quantification of genetically modified organisms, bacteria, and viruses [11, 19, 20], and can be of benefit also in plant health field. ddPCR is also a useful method for library quantification in next generation sequencing applications. In addition to the minimum information for publication of quantitative real-time PCR experiments (MIQE) [21], guidelines describing the minimum information for the publication of dPCR experiments have been also made available [9].

In this chapter we focus on the ddPCR workflow for pathogen (DNA or RNA) quantification using the Bio-Rad QX100 (or QX200) system, introducing also as an example the case of *E. amylovora*. In addition to the workflow we show examples of the different signals that can be obtained and give some hints on the data analysis.

2 Materials

2.1 Samples and Controls (See Note 1)

1. Extracted DNA or RNA from the plant or matrix under analysis (*see* **Note 2**).
2. Buffer extraction control. Buffer is added to the nucleic acid extraction procedure to monitor for potential contamination occurring during extraction.
3. Negative matrix control. Same material or matrix is added to the nucleic acid extraction, to assess the background signal inherent to the matrix.
4. No template control. Nuclease-free water used for master mix preparation is applied to the ddPCR reaction, to monitor for eventual contamination introduced during master mix preparation.
5. Positive control. A known concentration of the target sequence is added to the ddPCR reaction, to assess ddPCR performance.

2.2 ddPCR Reagents (See Note 3)

1. 2× ddPCR Super Mix for probes (Bio-Rad) for DNA amplification or 2× One-Step RT ddPCR Supermix kit (Bio-Rad) for RNA to cDNA transcription and amplification in one step (*see* **Note 4**).
2. Droplet generation oil for probes (Bio-Rad).
3. Molecular grade nuclease-free water.
4. Primers and probe.

2.3 Equipment

1. QX100 (or QX200) Droplet Digital PCR system (Bio-Rad) including droplet generator, droplet reader, and QuantaSoft data acquisition and analysis software (Fig. 1b, c).
2. Thermal cycler, i.e., T100 (Bio-Rad).
3. Droplet generator cartridge holder (Bio-Rad) (Fig. 1a).
4. DG8 droplet generator cartridges and gaskets (Bio-Rad, Cat. No. 186-4008 and 186-3009) (Fig. 1a).
5. Easy Pierce foil plate seals (Thermo, Cat. No. AB-0757).
6. 96-well PCR plate (Eppendorf Cat. No. 951020362).
7. PCR plate sealer (Eppendorf, Model No. 5390 000.024).
8. Set of pipettes. Manufacturer recommends Rainin (L-20, L-50, L8-50, L8-200) for the steps of droplet generation and droplet handling (*see* **Note 5**).
9. Tips for pipettes with aerosol barrier filters. Manufacturer recommends Rainin for the steps of droplet generation and droplet handling (*see* **Note 5**).
10. Microfuges and vortex for master mix preparation and appropriate nuclease-free plasticware.

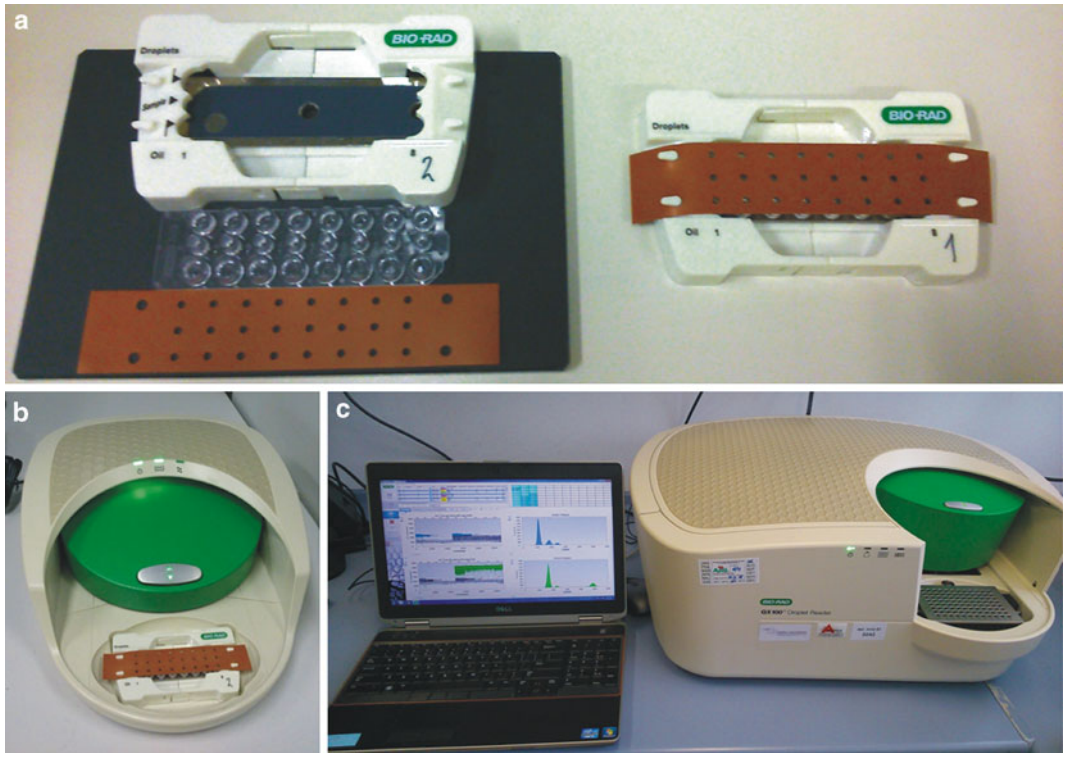

Fig. 1 (**a**) *Left side*: droplet generator cartridge holder, cartridge and gasket. *Right side*: assembled components prepared for droplet generation. (**b**) Droplet generator with inserted cartridge holder. (**c**) Droplet reader prepared for insertion of a 96-well plate and Quantasoft software

3 Methods

The workflow for detection and quantification of any RNA/DNA target using the QX100 system consists of four main steps: (1) preparation of the reaction mixture, (2) droplet generation, (3) PCR amplification, and (4) droplet reading and analysis of results.

3.1 Preparation of the Reaction Mixture

1. Mix, in nuclease-free tubes of appropriate volume, the concentrations of primers and probe for each assay with the corresponding 2× ddPCR master mix (Tables 1 and 2). Preparation of duplex or multiplex assays is also possible (*see* **Note 6**). Note that manganese acetate needs to be added in the case of the one-step master mix. Final reaction volume should be planned to 20 μl. Follow good laboratory practice in order to avoid contamination during mix preparation.
2. Before adding the sample, distribute the prepared mix into nuclease-free tubes, strips, or 96-well plates.
3. Add each sample (DNA/RNA samples and controls) into each of the tubes containing master mixes. Mix thoroughly by pipetting up and down, or by vortexing followed by a brief

Table 1
Reaction setup for DNA amplification in ddPCR

Component	Volume (μl)	Final concentration
2× ddPCR super mix for probes	10	1×
20× Target primers/probe (FAM)	1	1× (i.e., 900 nM/150 nM)[a]
20× 2nd Target primers/probe (VIC)[b]	1	1× (i.e., 900 nM/150 nM)
Nuclease-free water	Variable	–
DNA sample	Variable	50 fg to 100 ng[c]
Final volume	20	–

[a]Concentrations of primers and probe should be preferably optimized previously (*see* **Note 7**)
[b]Duplex assay can be included to monitor for a second target or reference gene (*see* **Note 6**)
[c]For quantification purposes the target concentration should be within the linear range of the method (*see* **Note 8**)

Table 2
Reaction setup for RNA amplification in one step RT-ddPCR

Component	Volume (μl)	Final concentration
2× one-step RT-ddPCR supermix	10	1×
Manganese acetate	0.8	1× (i.e., 900 nM/250 nM)
20× Target primers/probe (FAM)	1	1× (i.e., 900 nM/150 nM)[a]
20× 2nd Target primers/probe (VIC)[b]	1	1× (i.e., 900 nM/150 nM)[a]
Nuclease-free water	Variable	–
RNA sample	Variable	50 fg to 5 ng[c]
Final volume	20	–

[a]Concentrations of primers and probe should be preferably optimized previously (*see* **Note 7**)
[b]Duplex assay can be included to monitor for a second target or reference gene (*see* **Note 6**)
[c]For quantification purposes the target concentration should be within the linear range of the method (*see* **Note 8**)

centrifugation. Each tube should contain 20 μl of reaction mixture (*see* **Note 9**).

3.2 Droplet Generation

1. Place a DG8 droplet generation cartridge into the cartridge holder (Fig. 1a).
2. Transfer 20 μl of each prepared reaction mixture to each of the 8 wells indicated as "sample" in the droplet generation cartridge (*see* **Note 5**). There should be no empty wells left (*see* **Note 10**). Precautions should be taken not to form bubbles in the bottom

of the well, as they could interfere with the droplet formation. Add 70 µl of droplet generation oil, in all the wells indicated as "oil." Do not leave the oil bottle open for extended periods of time to avoid evaporation and stability of components. Hook the gasket over the cartridge holder using the holes in both sides.

3. Place the holder with the cartridge in the QX100 droplet generator unit (Fig. 1b). Initiate droplet generation. Oil and sample are pushed through microfluidic channels and mixed in the cartridge in the process forming droplets. Droplets are accumulated in the droplet well. The process takes 2–3 min for each cartridge.
4. Once droplets are generated the gasket is removed and 40 µl of the droplet suspension ("droplets" lane in the cartridge) are transferred from the cartridge to a 96-well PCR plate. The pipetting both for collecting the droplet suspension and for dispensing it in the PCR plate wells should be slow to protect the integrity of the droplets (*see* **Note 5**).
5. After all the samples (*see* **Note 11**) have gone through droplet generation and have been transferred to the 96-well PCR plate, the plate is heat sealed with a pierceable foil (*see* **Note 12**). Do not spin down the plate. This would break down the droplets.

3.3 PCR Amplification

1. Transfer the sealed plate to the thermocycler and run the PCR in the conditions shown in Table 3. Modifying the manufacturer provided cycling conditions can serve to improve the performance of certain assays (better cluster separation, higher specificity, *see* **Note 7**).

Table 3
PCR cycling conditions

	DNA samples			RNA samples		
Step	**T (°C)**	**Time**	**Cycle #**	**T (°C)**	**Time**	**Cycle #**
RT	–	–	–	60	30 min	Hold
Enzyme activation	95	10 min	Hold	95	5 min	Hold
Denaturation	94	30 s	40	94	30 s	40
Annealing and extension	60	1 min		60	1 min	
Heat deactivation	98	10 min	Hold	98	10 min	Hold
Hold	4	∞	Hold	4	∞	Hold

Ramp rates should be adjusted to 2–3 °C/s

3.4 Droplet Reading and Analysis of Results

1. Transfer the PCR plate to the QX100 droplet reader (Fig. 1c) and close the lid (*see* **Note 13**).
2. In the QuantaSoft software (Bio-Rad), click "Setup" and define the information for each well/sample, including name, type of experiment, type of sample and detectors or channels (FAM and/or VIC).
3. Click Run to start the reading. The droplet reader acts as a flow cytometer and reads each droplet to determine their signal and amplitude in the selected detectors.
4. Click "Analyze." The critical point is to set a threshold that allows the software to differentiate between negative and positive droplets. The software offers the possibility of defining this automatically or manually however, other approaches to analyses are available and may in specific cases be more suitable (*see* **Note 14**).
5. The software offers different ways of viewing the results, (1D amplitude of one channel, 2D amplitudes of both channels, copy number in each well/channel …) that are more or less informative depending on the type of experiment (absolute quantification, allele discrimination, multiplexing, …). It ultimately gives a table with parameters resulting from the analysis, such as the concentration of target copies/microliter of reaction, the number of total accepted droplets, the positive ones and the negative ones (*see* **Note 15**).

3.5 Case Study, Quantification of the Quarantine Pathogen E. amylovora

As a practical example, we describe a recently published ddPCR based protocol for the quantification of the DNA quarantine bacterial pathogen *E. amylovora* [18] (Table 4 and Fig. 2). The method was transferred from a previously published qPCR assay [22]. There are also examples in the literature of one step RT-ddPCR assays that target RNA pathogens, such as Rotavirus [10] and Pepper Mild Mottle virus ([12] and Fig. 4).

1. DNA from the pure bacterial cultures and plant extracts was extracted using magnetic bead based QuickPick™ SML Plant DNA kits (Bio-Nobile, Turku, Finland) in a King Fisher™ automated system (Thermo Labsystem) as described previously for *E. amylovora* [22], and with a minor modification (440 µl lysate used in the purification).
2. Reaction components are mixed as shown in Table 1 with 4 µl of DNA in a final 20 µl volume. Concentrations of primers and probe are the same as the previously optimized by Pirc et al. for the qPCR assay, 900 nM and 250 nM, respectively [22]. For droplet generation and PCR, the same steps and cycling conditions as the ones described above for a DNA target were followed.

Table 4
Selected parameters calculated for the *E. amylovora* samples

Sample	Positive droplets	Accepted droplets	λ	Quantity-Reaction (copies)	P	Effective reaction size	Positive mean	Negative mean
A09	15,699	15,708	7.5	117,255.50	0.999	71.5	12,716.00	2,060.70
B09	13,728	13,757	6.2	84,770.70	0.998	62.6	12,700.80	1,997.30
C09	12,122	12,881	2.8	36,472.60	0.941	58.6	12,700.80	1,873.90
D09	2,832	13,042	0.2	3,192.80	0.217	59.3	12,544.30	1,814.40
E09	532	12,464	0	543.7	0.043	56.7	10,382.70	1,760.70
F09	36	15,398	0	36	0.002	70.1	11,769.40	1,767.10
G09	8	14,110	0	8	0.001	64.2	7,404.90	1,782.70
H09	3	15,491	0	3	0	70.5	2,547.30	1,788.50

Parameters were calculated using an R script [18] for the positive samples shown in Fig. 2 and include parameters listed in the minimum information for the publication of dPCR experiments as previously described [9]. The highest concentration shown corresponds to 3×10^7 target DNA copies per mL. *See* [18] for calculation of additional parameters describing the quality of separation of positive and negative droplets

Parameters: Positive drop = number of positive droplets, Accepted drop = number of accepted droplets, λ = mean copies per partition, calculated using the number of positive partitions, as described in Huggett et al. [9]: $-\ln(1 - (\text{number of positive partitions})/(\text{number of accepted droplets}))$, Quantity-Reaction(copies) = target concentration expressed in copies per ddPCR reaction, P = fraction of positive droplets (number of positive droplets)/(number of accepted droplets), Effective reaction size = total volume of partitions measured. It is calculated by multiplying the number of accepted droplets with the volume of partition. The volume of droplets is assumed to be 0.91 nL, consistent with the instrument manufacturer's software; Positive mean = mean of the signal in positive droplets and Negative mean = mean of the signal in negative droplets

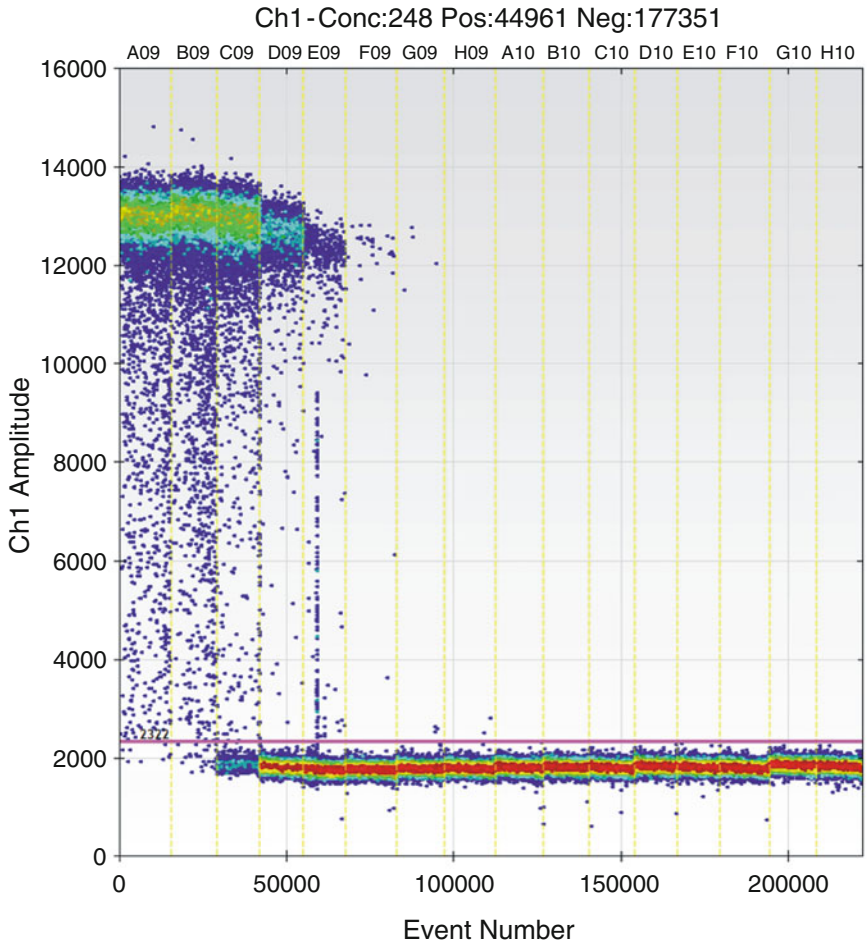

Fig. 2 Results of amplification of *E. amylovora* DNA in ddPCR. Serial tenfold dilution of *E. amylovora* DNA (A09 to H09) and no template controls (A10 to H10) are shown. For each sample droplets are depicted according to the event (number of droplet as read during reading) and its fluorescence (Ch1 Amplitude). The concentration of droplets is seen as a heat map with the highest concentration depicted in *red* and the lowest in *blue*. At higher concentrations of the target DNA the number of negative droplets is *low* and the test is approaching its *upper limit* of the dynamic range. Note the distribution of positive droplets over a range of fluorescence values that is caused by small differences in the amplification efficiencies or delayed amplification (Monte Carlo effect) and the absence of positive droplets in no template controls. The threshold discriminating between negative and positive droplets is set at fluorescence of 2,322 and was chosen as previously described [18]. Because of the Poisson modelling that is used for calculating concentration, different threshold have little effect on the concentrations however they can have pronounced effect on the plus/minus detection. Concentrations determined and parameters calculated for the positive samples shown here are listed in Table 4

3. After thermal cycling the plate is transferred to the droplet reader. The software package (Quanta Soft) from Bio-Rad was used for data acquisition and analysis.
4. A manual threshold value of 2.322 was defined [18], after evaluation of different types of negative samples (no template

controls and negative plant material) and positive samples (plant material containing low concentrations of target bacteria and serial dilutions of target DNA). The mentioned threshold value allowed proper detection and quantification and of all the tested samples. The automatic threshold assignment by the software yielded very similar results to the manual threshold, as well as the use of the "Defining the rain" tool (http://definetherain.org.uk/). Therefore, for this particular assay any of these three strategies can be used for data analysis however, in particular with suboptimal assays, the analysis approach can significantly influence the results [18].

5. In Fig. 2 examples of the results obtained with different samples are shown: NTC, negative matrix, low target concentration, and high target concentration. In Table 4 the ddPCR parameters corresponding to each of the samples from Fig. 2 are shown: total accepted droplets, positive droplets, negative droplets, concentration, and average number of copies per partition (λ). The QuantaSoft software gives a list of most of these parameters in tabular form. Dreo et al. [18] developed an open source R script that allows automation of the ddPCR data analysis under different settings and calculates additional important data (i.e., λ), thus significantly simplifying the optimization of this step and allowing for high-throughput analysis of samples (*see* **Note 15**).

4 Notes

1. Depending on the nature of the ddPCR experiment, all of the mentioned controls may not be necessary, or alternatively, some controls that have not been listed here may be needed, but this would be subject of another chapter. Diagnostic applications are likely to require more controls than research ones. For example useful controls consist of spiking a negative sample with target pathogen at concentrations close to the limit of detection and process it in parallel to other samples through DNA extraction and PCR. Assay optimization runs will also require additional controls, in order to evaluate the different signal amplitudes that are generated and associate those with positive and negative droplets. It is not uncommon to observe a small number of droplets [1–3] with higher amplitude than the negative droplets in negative samples. Sometimes the amplitude of such droplets can equal the one of the real positives. In this case based on negative sample observations a minimum number of droplets to make a sample positive should be defined. A previous report defined two droplets as minimum for considering a sample positive [18].

2. For RNA or DNA isolation same protocols or kits as those used for qPCR can be used for ddPCR. The higher resilience of ddPCR to inhibitors reported in several recent studies [10–12, 23] allow the use of a broader range of nucleic acid purification methods, including more simple extraction protocols. The possibility of direct ddPCR analysis and quantification of bacteria without DNA extraction has been also confirmed [18].
3. At present, the Bio-Rad platform requires the use of their proprietary master mixes enabling stable droplets formation, while platforms based on partitioning into chambers allow for testing different commercially available PCR mixes.
4. In the case of analysis of an RNA pathogen in a two-step format, a reverse transcriptase (RT) or RT kit with confirmed optimal performance should be selected. For the RT optimizations known concentrations of the RNA target, i.e., an in vitro transcript quantified spectrophotometrically, should be applied to different RT systems, and the yield of the RT estimated. The RT yield should preferably be as close as 100 % as possible, otherwise the quantification with ddPCR requires a correcting factor to be genuinely absolute.
5. The highest accuracy when pipetting is required. In addition, when handling the droplets, the pipetting should be done very carefully to preserve their integrity. For example when transferring the freshly generated droplets from the cartridge to the PCR plate, the pipetting should be executed at slow and constant speed, taking 10 s both for the collection and dispense. The use of regularly calibrated pipettes that ensure an accurate liquid handling is highly recommended. Bio-Rad, for example, recommends using Rainin pipettes (single and multichannel).
6. The QX100 and QX200 systems allow duplexing with fluorescence data acquisition in both the FAM and HEX-VIC spectral regions, respectively. There is increasing number of studies where multiplexing with ddPCR has been reported [11, 19, 20, 24]. In addition, multiplexing of up to four assays using the same reporter dye has been demonstrated by optimizing primer and probe concentrations that lead to positive signals of different fluorescence intensity. QX100 is not compatible with the use of intercalating dyes such as SYBR Green or Eva Green, but this issue has been addressed in the newer model QX200.
7. Assays (primer and labeled probe) for dPCR are designed in the same way as for qPCR and same tools can be used (i.e., Primer Express from life Technologies, or others). Assays that have been optimized in qPCR format are easily transferred to ddPCR format [1, 10, 11, 17, 18], while assays that have shown limitations in qPCR (cross-amplification, low efficiency

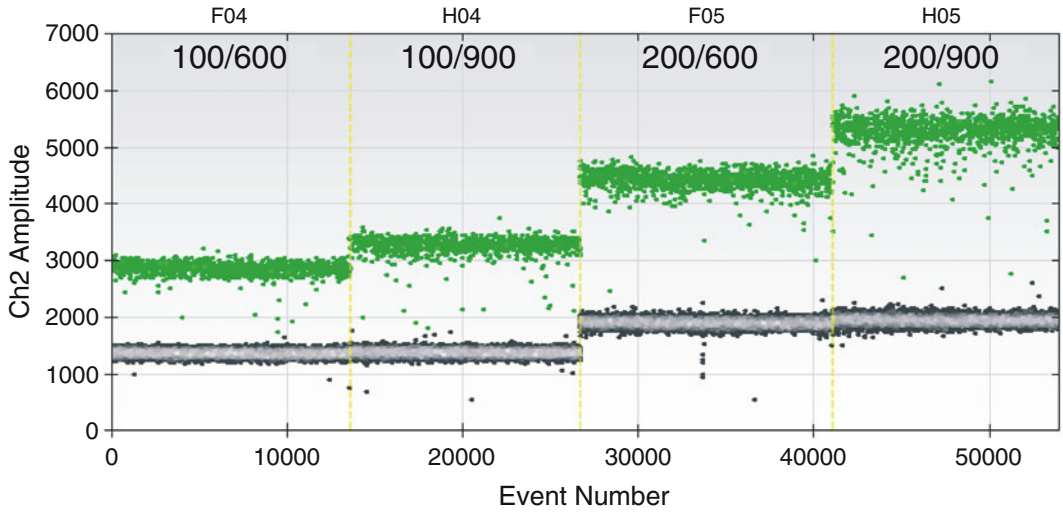

Fig. 3 Influence of primer and probe concentration. ddPCR analysis of the same viral target using the same assay with four different primer/probe concentrations (100 nM/600 nM, 100 nM/900 nM, 200 nM/600 nM, 200 nM/900 nM). Increase of primer concentrations results in an increase in the amplitude of positive droplets. Increase in the probe concentrations results in an increase in the amplitude of both positive and negative droplets. With the highest primer/probe concentrations the highest separation between positive and negative droplets is achieved but an increase in the dispersion (rain effect) of the positive droplets is observed. Optimal primer/probe concentration for this particular assay was 200 nM/600 nM

of amplification) are more likely to fail in a ddPCR assay as well [18]. Note that the closer the cycling protocol of qPCR is to the cycling protocol in ddPCR, the easier the transfer of assays. Primers and probe concentrations have an influence on the amplitude and clustering of the droplets (*see* Fig. 3), and therefore, testing different concentrations of primers and probe may be a wise thing to do in case the assays do not show the desired performance in ddPCR format. Annealing temperature is also a critical point that offers space for optimization and changing other cycling conditions such as elongation time or cycle number. Additional validation study includes testing of the matrices where the pathogen of interest will be detected/quantified to assess the background signal amplitude from the matrix and for any cross-reaction [18]. Testing of a higher number of NTC of a given assay is also necessary to evaluate the possibility of false positive results. All this information will be of help when deciding on the threshold cutoff (*see* **Note 14**).

8. Poisson distribution accounts for the presence of more than one target copy inside a droplet but a minimum number of negative droplets are necessary in order to calculate the target concentration. The theoretical upper limit of the linear range is defined by the number of available partitions (droplets in this case). For this reason, to achieve accurate quantification, if a

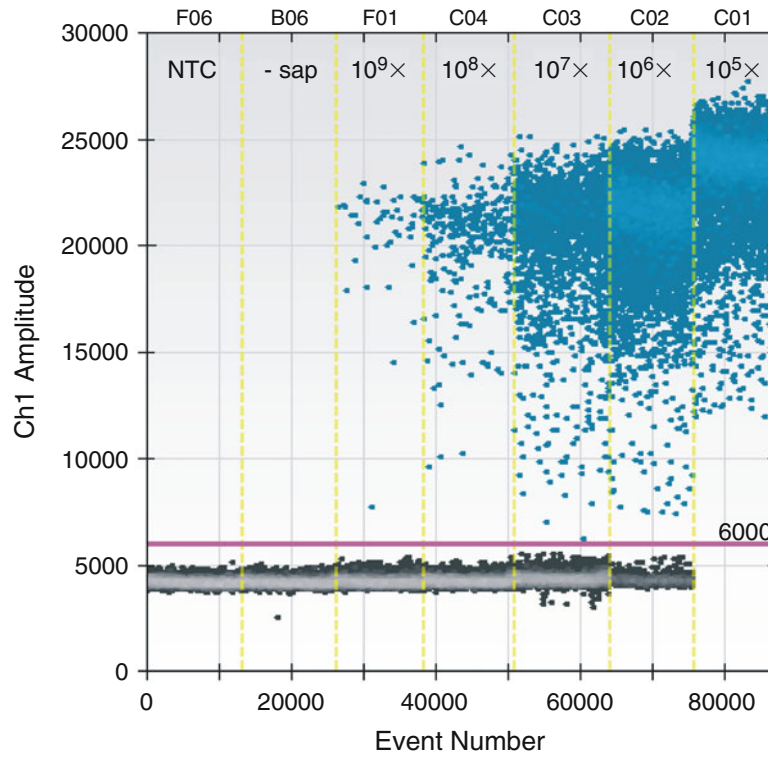

Fig. 4 Dose dependence of ddPCR. Tenfold dilution series of *Pepper mild mottle virus* (PMMoV) infected plant sample analyzed in one step RT-ddPCR format. The clusters of negative and positive droplets are easily recognized. A manually set threshold separates both types of droplets. Droplets associated to the "rain" effect observed in some of the dilutions were considered positive in this experiment after evaluation of droplet amplitudes in negative samples. *NTC* non template control, *sap* healthy plant sap

saturation of the droplets is observed, the target should be diluted to a concentration within the linear range of the method (*see* Fig. 2 A09 and B09 samples and Fig. 4, 10^5-fold diluted sample). The theoretical dynamic range of ddPCR is broad and up to 100,000 copies per reaction can be quantified which is higher than the commonly examined concentrations of plant pathogens in samples.

9. When preparing the master mix it is wise to account for excess volume for each component in order to ensure the presence of at least 20 μl in the reaction mixture before transferring it to the droplet generator cartridge.
10. If there are not enough samples as to fill all of the wells with the reaction mixture a special buffer (ddPCR™ Buffer Control Kit—Bio-Rad) should be used to fill the remaining wells. In spite of this we recommend that experiments are designed in a way that all the wells are filled with reaction mix, so that DG8 droplet generation cartridges are used at their full capacity.

11. The process of droplet generation is repeated as many times as required by the sample number. When longer preparation times are expected due to high number of samples it is recommended to keep both, samples and droplet suspensions, at 4 °C using refrigerated blocks.
12. Plates should only be sealed when the plate sealer has reached its optimal temperature. In our experience, pressure to the plate with the heated sealer should be applied 2× for 5 s. The foil on the plate should be inspected for the presence of circles from the wells beneath the foil. If the pressure is applied for too long the seal will be broken and all the oil and the reaction mixture will evaporate during PCR reaction.
13. In the case that the droplet reader is unavailable after PCR cycling, the plate can be stored at 4 °C for several hours or even overnight.
14. The variation of signals depends on many factors, e.g., assay characteristics [18], matrix or presence of inhibitors [10], single nucleotide polymorphisms (SNP) in the probe annealing region (Fig. 5) and others. All these factors can affect the separation between negative and positive droplets in different ways, resulting for example in the presence of higher signal droplets in negative samples or in the induction of the so-called droplet rain effect (Figs. 2 and 4). Therefore, in certain cases automatic analysis can be sometimes misleading, and setting the threshold manually can be necessary. An example of the influence of a single nucleotide polymorphism (SNP) in the probe annealing region is shown in Fig. 5. In this example wild type and mutant assays differ by one SNP in the probe. When wild type DNA is applied to mutant specific assay and vice versa, positive droplets with signal amplitude higher than the negative ones are observed due to cross-reactivity. Automatic analysis considers the cross-reactive droplets as positives (Fig. 5 upper panels), by defining manually the threshold can assign those amplitudes as negative (Fig. 5 lower panels). Observed cross-reactivity could also be improved by modifying the assay (annealing temperature, cycle number, …) or redesigning it (LNA probes, SNP in the 5′, …). Manual definition of the threshold can also help overcoming other droplet particularities, such as rain effect. Both in diagnostic and quantitative applications, it is important to evaluate the signal amplitude obtained with negative samples (NTC, isolation controls, matrix controls, closely related pathogens). The threshold can be then manually defined to consider such signals as negative. For correctly designed assays that perform optimally automatic analysis is sufficient. A comprehensive study showing different ways of analyzing the ddPCR data based on a case study with two assays targeting two bacterial plant pathogens has been

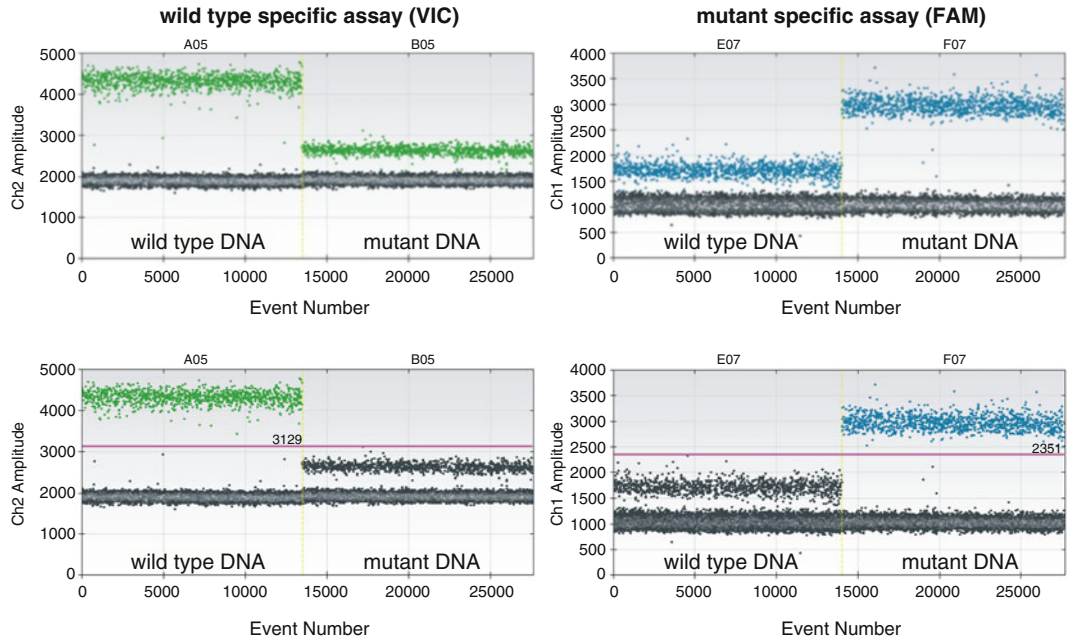

Fig. 5 Effect of single nucleotide polymorphisms. Analysis of a wild type and mutant viral sequences using two assays specific for the wild type (FAM) and mutant (VIC) sequences, respectively. Note that the mutant sequence still cross-reacts with the wild type assay and similarly the wild type sequence cross-reacts with the mutant assay (*upper panels*). An optimal separation of the clusters of droplets with specific signal and cross-reactive signal was achieved, allowing a correct quantification by manually setting the threshold (*lower panels*). The manual threshold was set at the lowest possible value that resulted in 0 positive droplets when applying mutant sequence to wild type assay and vice versa

recently published [18]. Automatic analysis was compared with manual threshold definition and the Web-based tool "definethereain" by challenging all three to the correct assigning of known positive and negative samples. Results show that the choice of the analysis depends on each particular assay's performance and characteristics. More detailed information on the stringency and characteristics of each analysis tool can be found in ref. [18].

15. The data obtained from the results table (concentration, number of droplets: total, negative, positive, accepted, …) can be used to calculate and optimize different parameters (Table 4) that are requested in the dPCR MIQE guidelines [9]. Some of these parameters are the mean number of copies per partition (λ), the individual partition volume, or the effective reaction size [9]. An R-based script that automates these calculations has been recently described [18]. Low number of total accepted droplets can negatively affect the precision of the result and, thus, several published studies define a limit of 10,000 accepted droplets, below which the result in that well is rejected [10, 11, 18].

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