Chapter 5

Digital PCR: Principles and Applications

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Abstract

Digital PCR is a robust PCR technique that enables precise and accurate absolute quantitation of target molecules by diluting and partitioning of the samples into numerous compartments. Automated partitioning can be attained by creating "water-in-oil" emulsion (emulsion-based digital PCR) or using a chip with microchannels (microfluidics-based digital PCR). We discuss the advantages and a wide variety of clinical applications of this technique. We describe the droplet digital RT-PCR protocol published by Jennings et al. for identification and absolute quantitation of BCR-ABL1 transcripts.

Key words Droplet digital PCR, Emulsion PCR, Microfluidics, BCR-ABL1 transcript

1 Introduction

Digital PCR is a robust PCR technique that enables accurate absolute quantification of target molecules at a high degree of sensitivity. It essentially combines the simplicity of traditional end-point PCR and the quantification features of the real-time quantitative PCR (qPCR) methodologies. Unlike qPCR, the quantitation is absolute and does not utilize calibration of standards, thus making the process faster, more precise, and reproducible [1].

The basic principle involves extreme limiting dilution and partitioning of the sample into millions of separate units that ideally contain either no particle or a single particle. Each unit contains all the reagents necessary for a PCR reaction, and basically functions as a micro-PCR reactor. If the unit contains the template of interest, PCR amplification yields a positive signal. If there is no template, there is no signal. The quantitation is binary, hence the term "digital." If the number of partitioning units is known, the initial amount of target molecules can be estimated by the knowledge of the total number of positive to negative signals [2]. At an extreme dilution, this simply represents the ratio of the positive to negative units as there is either no template or a single template within the unit. At a higher concentration, to account for the possibility of

Rajyalakshmi Luthra et al. (eds.), *Clinical Applications of PCR*, Methods in Molecular Biology, vol. 1392,
DOI 10.1007/978-1-4939-3360-0_5,

multiple target molecules in each unit, an adjustment factor using Poisson distribution analysis is incorporated into the analysis. In such a case, the number of target molecules can be determined using the equation $\lambda = -\ln(1-p)$, where λ is the average number of target molecules per unit and p is the ratio of the number of units with positive signal to the total number of partition units. The absolute target concentration can be estimated using λ, the reaction volume in each of the units, and the total number of partition units. It is apparent that the larger number of partitions, the higher the precision and the dynamic range of the assay [3].

Traditionally, digital PCR was an elaborate procedure that required conducting serial dilutions of the sample followed by PCR amplifications. Currently, advanced techniques can be conveniently used for automated partitioning of the sample of interest into numerous (up to 100,000) known number of separate micro-PCR reactors designed to amplify the target of interest [4]. Most instruments commonly employ one of the two modalities of partitioning. The first is based on emulsion chemistry which involves creating emulsions of aqueous droplets in an oil medium, where each droplet functions as a PCR unit (known as droplet digital PCR, ddPCR) [5, 6]. The second involves microfluidics where a physical microchannel (either surface or capillary based) system is designed on plates or arrays to create partitions [7, 8]. Emulsion-based digital PCR systems allow analysis of a greater range of sample concentrations for any given precision than chip-based systems due to a much larger number of partitions [4].

There are several distinct advantages of digital PCR compared to other types of PCR techniques. Digital PCR can detect target molecules at a much lower frequency (in 100,000) than qPCR [3]. In fact, the sensitivity in this is limited only by the number of molecules present in the sample that can be amplified [5]. With regard to copy number analysis, it has been shown that changes less than 1.2-fold difference can also be detected by digital PCR [9]. As mentioned before, digital PCR provides an absolute quantitation of nucleic acids without the need for an external standard, thus making it faster, more precise, and reproducible than qPCR [2]. Since quantitation involves determination of the presence or absence of amplification signal in each of the multiple units, similar to an end-point PCR, it is less dependent on the factors affecting PCR amplification efficiency such as the presence of inhibitors, target/primer accessibility issues due to poor denaturing, or point mutation on the primer or probe annealing site of the target molecule [10]. Digital PCR technique facilitates easier design of multiplexing for assessment of multiple targets. Use of traditional multiplex qPCR can be a challenge for measurement of multiple targets when there is marked difference between various target concentrations leading to preferential amplification of high-abundance target molecules by depletion of reagents. Digital PCR

overcomes this challenge since each individual micro-reaction is generally positive for one of the templates. This has been aptly termed "synthetic enrichment effect" [10].

Digital PCR has already been employed in a variety of applications in cancer diagnostics that include detection of rare mutations [3, 7, 8], copy number analysis, specifically for HER2 and EGFR [3, 9, 11–16], as well as absolute quantitation [17–20]. In addition, it has been widely used in microbiological identification and quantification of bacteria [21] and viruses [22–24]. Further, digital PCR has been shown to be more sensitive than qPCR in cell-free fetal DNA testing for paternally inherited single-gene disorders [25, 26]. For efficient adoption and utilization of this technology by the scientific community in general, minimum experimental parameters to be indicated in the protocols are available [4].

Jennings et al. have shown that the droplet digital RT-PCR offers a much simpler interface for identification and quantitation of BCR-ABL1 transcripts, with an improved lower limit of detection and absolute quantification without bias [18]. Using their technique, the sample is partitioned into 20,000 nanodroplets that are individually amplified by PCR. This is followed by identification of the number of positive and negative droplets for BCR-ABL1 fusion transcript, BCR transcript, and both by fluorescence detectors. Here, we describe the protocol used by the authors for identification of BCR-ABL1 transcripts using digital PCR and based on the recommendations from the manufacturer (Bio-Rad). Briefly, the steps involved include cDNA generation, droplet generation, PCR, droplet reading, and analysis.

2 Materials

1. Primers and probes.

   TaqMan MGB probes and primers (Life Technologies, Carlsbad, CA) generated for the analysis are as follows: BCR-ABL1 (forward) 5′-CATTCCGCTGACCATCAATA-3′; BCR-ABL1 (reverse) 5′-ACACCATTCCCCATTGTGAT-3′; BCR-ABL1 probe 5′-/56-FAM/CCCTTCAGCGGCCAGTAGC ATCTGA-3′; BCR (forward) 5′-CAGTGCGTGGAGGAGA TCGA-3′; BCR (reverse) 5′-CGATGCCCTCTGCGAAG TTG-3′; and BCR probe 5′-VIC-CAGCCTTCGACGTCAA-3′.
2. Input RNA.
3. Nuclease-free water.
4. One-Step RT-ddPCR kit from Bio-Rad Laboratories (Cat# 1863021; Hercules, CA).
5. Heat block *or* water bath.
6. 96-Well PCR plates.

7. Pipets.
8. Pipet tips.
9. Centrifuge.
10. QX100 droplet generator (Bio-Rad).
11. DG8 droplet generator cartridges (single-use; Bio-Rad).
12. DG8 droplet generator cartridge holder (Bio-Rad).
13. DG8 gaskets (single-use; Bio-Rad).
14. ddPCR droplet generation (DG) oil (Bio-Rad).
15. Bio-Rad QX100 droplet reader.
16. Droplet reader plate holders.
17. QuantaSoft software.
18. Eppendorf twin.tec semi-skirted 96-well plate.
19. Heat sealer.
20. Heat sealing plate foil.
21. Thermal cycler.

3 Methods (Adapted from Jennings et al. [18])

1. Incubate the RNA samples for 5 min at 75°.
2. Thaw the components of the One-Step RT-ddPCR kit from Bio-Rad Laboratories (Cat# 1863021; Hercules, CA) at room temperature. The contents of each of the reagents are adequately mixed by either pipetting or inverting followed by centrifugation.
3. Prepare the RT-PCR reaction mixtures for BCR and BCR-ABL1 transcripts as detailed in Tables 1 and 2.

Table 1
RT-ddPCR reaction mix for BCR transcript

Component	Volume per reaction	Final concentration
2× One-step RT-ddPCR supermix	10 µL	1×
25 mM manganese acetate solution	0.8 µL	1 mM
Forward BCR primer		900 nmol/L
Reverse BCR primer		900 nmol/L
BCR probe		250 nmol/L
RNase/DNase-free water		
RNA template	40 ng	
Total volume	20 µL	

Table 2
RT-ddPCR reaction mix for BCR-ABL1 transcript

Component	Volume per reaction	Final concentration
2× One-step RT-ddPCR supermix	10 µL	1×
25 mM manganese acetate solution	0.8 µL	1 mM
BCR-ABL1 forward primer		2250 nmol/L
BCR-ABL1 reverse primer		2250 nmol/L
BCR-ABL1 probe		625 nmol/L
RNase/DNase-free water		
RNA template	600 ng	
Total volume	20 µL	

4. Dispense equal amounts of the reaction mixtures to each of the reaction tubes and then add appropriate amounts of the template. Mix either by pipetting or vortexing followed by centrifugation and let it stand at room temperature.
5. Insert the DG8 cartridge into the holder. The notch in the cartridge should be located at the upper left side of the holder.
6. Pipet 20 µL of droplet digital PCR (ddPCR) reaction mixture from eight samples at a time (make sure it is at room temperature) using eight-channel pipet and load it into wells designated as "sample" (middle row) on the Bio-Rad DG8 disposable droplet generator cartridge. It is important to avoid air bubbles while pipetting that may interfere with the droplet generation (*see* **Note 1**).
7. Pipet 70 µL of droplet generation oil into each of the wells designated "oil" (bottom row) for each of the samples on the cartridge.
8. All eight samples designated "samples" must contain either sample of 1× buffer control. All eight samples designated oil should contain droplet generator oil.
9. Firmly attach a new rubber gasket over the cartridge holder (*see* **Note 2**).
10. Place the cartridge holder in the QX100 droplet generator, and close the lid.
11. Droplet generation is completed in about 2 min as indicated by all the three lights on the machine turning solid green.
12. Remove the holder with the cartridge still in place from the QX100. Removing the gasket, and inspect. The sample and oil wells should be almost empty. The droplets should be present in the "droplet wells" (top row) and should be slightly opaque.

13. Transfer 40 μL from each of the wells with generated droplets gently to an Eppendorf 96-well twin.tec PCR plate (Eppendorf, Hamburg, Germany) using multichannel micropipette (*see* **Note 3**).
14. Immediately cover the contents of the wells to prevent evaporation.
15. After all the samples have been loaded, heat-seal the plate with a Thermo Scientific Easy Pierce pierceable foil seal from Thermo Fisher Scientific (Cat#AB-0757; Waltham, MA). Avoid centrifugation once the droplets are generated.
16. The cartridge can then be discarded from the holder.
17. PCR should be performed within half hour of sealing the plate. If not, the plate can be stored for up to 4 h at 4 °C. The plate is placed on a thermal cycler and amplified to the endpoint at conditions indicated below:

    60 °C × 30 min (1 cycle), 95 °C × 5 min (1 cycle), 95 °C × 30 s (ramp rate 2.5 °C/s), and 59 °C × 60 s (ramp rate 2.5 °C/s) (40 cycles), 98 °C × 10 min (1 cycle), and a 12 °C hold.
18. Secure the PCR plate on the PCR plate holder.
19. Turn on the QX100 droplet reader and launch the QuantaSoft analysis software version 1.3.2.0. It is important to make sure that the droplet reader has sufficient droplet reader oil in the supply bottle and less than 700 mL in the waste bottle.
20. Load the 96-well PCR plate on the holder into the Bio-Rad's QX100 droplet reader and close the lid.
21. Set up a new experiment with the sample details on the QuantaSoft analysis software and start the run. Individual droplets are analyzed as positive and negative by a two-color detector to provide absolute quantification in digital form. Remove the PCR plate and the holder and discard the PCR plate.
22. Once the run is completed, data can be analyzed.

4 Notes

1. According to the Bio-Rad manual, air bubbles, even when not visible, prevent the samples from reaching the bottom of the wells and into the microchannels, thereby significantly reducing the amount of droplets. They recommend the use of 20 μL aerosol-barrier (filtered) Rainin pipet tips and not 200 μL pipette tips. Instead of pipetting the sample directly on the side of the well, they recommend gently sliding the pipet down the side wall at a 15° angle past a ridge near the bottom. Gently pipet half the sample at 15° angle at this location, and dispense the rest while sliding the pipette back up the wall.

2. The gasket must be securely attached to ensure sufficient pressure for droplet generation.
3. For transferring the droplets from the cartridge onto the PCR plate, Bio-Rad recommends the use of eight-channel manual P-50 Pipetman with 200 μL tips (not wide or narrow bore), and not the P-20 or P-1000 Pipetman. With the cartridge holder being flat, they suggest aspirating 40 μL of the droplets (not more) with the pipet at a 30–45° angle from the vertical direction at the junction of the side wall and the bottom. The droplets should be dispensed near but not at the bottom of the wells of the PCR plate slowly.

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