Chapter 5

Minimal Residual Disease Analysis by Monitoring Immunoglobulin and T-Cell Receptor Gene Rearrangements by Quantitative PCR and Droplet Digital PCR

Irene Della Starza, Cornelia Eckert, Daniela Drandi, and Giovanni Cazzaniga and on behalf of the EuroMRD Consortium

Abstract

Analysis of immunoglobulin and T-cell receptor gene rearrangements by real-time quantitative polymerase chain reaction (RQ-PCR) is the gold standard for sensitive and accurate minimal residual disease (MRD) monitoring; it has been extensively standardized and guidelines have been developed within the EuroMRD consortium (www.euromrd.org). However, new generations of PCR-based methods are standing out as potential alternatives to RQ-PCR, such as digital PCR technology (dPCR), the third-generation implementation of conventional PCR, which has the potential to overcome some of the limitations of RQ-PCR such as allowing the absolute quantification of nucleic acid targets without the need for a calibration curve. During the last years, droplet digital PCR (ddPCR) technology has been compared to RQ-PCR in several hematologic malignancies showing its proficiency for MRD analysis. So far, no established guidelines for ddPCR MRD analysis and data interpretation have been defined and its potential is still under investigation. However, a major standardization effort is underway within the EuroMRD consortium (www.euromrd.org) for future application of ddPCR in standard clinical practice.

Key words Minimal residual disease, Immunoglobulin, T-cell receptor, Rearrangement, RQ-PCR, ddPCR

1 Introduction

After a single lymphoid cell undergoes clonal neoplastic transformation, all progeny leukemic cells will contain the same rearranged clonal Immunoglobulin (IG) and T-cell receptor (TR) genes, thus representing highly specific molecular targets for minimal residual disease (MRD) detection in lymphoproliferative disorders [1].

MRD monitoring has been proven to be a compelling tool for advising therapeutic choices especially in acute lymphoblastic leukemia (ALL), the first neoplasm where MRD has been used to assess early response to therapy [2–6]. The availability of drug combinations capable of unprecedented complete clinical responses

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leads to a growing interest for MRD assessment also in other lymphoid malignancies over time, i.e., chronic lymphocytic leukemia, multiple myeloma, as well as mantle cell lymphoma [7–10].

Currently, antigen-receptor gene analysis by real-time quantitative polymerase chain reaction (RQ-PCR) is the gold standard for sensitive and accurate MRD monitoring and has been extensively standardized within the EuroMRD consortium (www.euromrd.org), which established guidelines for the analysis and interpretation of RQ-PCR data [11] to favor a homogeneous application of MRD studies within different lymphoid malignancies and treatment protocols all over the world. However, the measurement of a dynamic process, such as the rate of target amplification, carries some intrinsic fluctuations that cannot be fully eliminated. The digital PCR technology (dPCR) [12], the new generation of conventional PCR, is based on partitioning by nanofluidics and emulsion chemistries which allow performing a limiting dilution of DNA into individual (partitioned) PCR reactions. The DNA template can thus be randomly distributed and the Poisson statistics can be applied to quantify the DNA amount in positive partitions. In comparison with RQ-PCR, dPCR allows the quantification of nucleic acid targets without the need of calibration curves [13]. Moreover, it has the potential to overcome some of the limitations of RQ-PCR. Based on the dynamic nature of these two methods, dPCR appears more accurate than RQ-PCR with a greater amplification efficiency, since each sample is partitioned and each partition is analyzed individually, so small changes in fluorescence intensity are more readily detected [14, 15].

Recently, droplet digital PCR (ddPCR) technology, a type of dPCR characterized by partitioning the sample in droplets, has been applied in comparison to RQ-PCR in several hematologic malignancies, and its additional technical and clinical value to the gold-standard RQ-PCR was demonstrated [16–22]. However, no established guidelines for ddPCR MRD analysis and interpretation have been defined so far, and its potential is still under investigation. A major standardization effort is underway within the ddPCR group of the EuroMRD consortium (www.euromrd.org) for its future application in standard clinical practice.

The PCR approach for IG/TR screening and RQ-PCR MRD analysis have been recently described in this book series, on behalf of the EuroMRD consortium [23]. Briefly, to identify IG/TR markers at diagnosis, either a standard multiplex-PCR/Sanger sequencing [23] or the new and more efficient NGS-based approaches [24, 25] can be applied to define the unique V-(D-)J junctional regions. Complementary patient- and allele-specific oligonucleotide (ASO) primers and common fluorescent probes must be designed for each target of any patient for its MRD monitoring. To perform the MRD relative quantification by RQ-PCR,

amplification conditions and sensitivity testing for each ASO-primer are established on the diagnostic material serially diluted in normal mononuclear cells, before quantifying MRD in bone marrow samples collected during treatment. Interpretation guidelines developed and continuously refined within the EuroMRD group are fundamental for issuing comprehensive clinical reports and for comparing independent studies applying the IG/TR RQ-PCR MRD monitoring [11].

Since the PCR approach for IG/TR screening and RQ-PCR MRD analysis in lymphoproliferative disorders has been recently described [23], in this chapter we will focus on the ddPCR protocol.

2 Materials

1. Supermix for probes (no dUTP).
2. Albumin gene primers and probe.
3. *HINFI (optional) (see below and notes)*.
4. Target gene primers and probe.
5. Thermal cycler.
6. ddPCR droplet generator.
7. PCR plate sealer.
8. ddPCR droplet reader.
9. PC and software for analysis of ddPCR data (Quantasoft, Bio-Rad Laboratories, Hercules, CA, USA) (note: not available for Mac).
10. DG8 cartridges, DG8 gaskets, ddPCR droplet generation oil for probes, ddPCR 96-well plates, pierceable foil heat seals, ddPCR droplet reader oil (Bio-Rad Laboratories).
11. QX100 or QX200 System.
12. 96-well PCR plates and optical adhesive films or 0.2 ml strip tubes with cups (used for mix preparation and collection before droplets generation).
13. Oligo Analyzer 3.1 (www.eu.idtdna.com).
14. PrimerQuest (Integrated DNA Technologies, www.idtdna.com).
15. Primer3Plus (www.primer3plus.com).

Along this chapter, the Bio-Rad system (Bio-Rad Laboratories) is described. Alternative instruments will require adaptation of this protocol.

3 Methods

To identify IG/TR markers at diagnosis, either a standard multiplex PCR/Sanger sequencing [23] or the new and more efficient NGS-based approaches [24, 25] can be applied to define the unique V-(D-)J junctional regions. Complementary ASO primers and common fluorescent probes must be designed for each target of each patient, for MRD monitoring [23]. Several tools are available for assay design and optimization, such as Oligo Analyzer 3.1 (www.eu.idtdna.com), PrimerQuest (Integrated DNA Technologies, www.idtdna.com), Primer3Plus (www.primer3plus.com), or others.

3.1 ddPCR MRD Quantification for the Target Genes

No standard curve generation is needed for a ddPCR experiment setup. However, as for any kind of PCR experiments, a positive control is mandatory (i.e., either a 10-1 dilution or 10-4 dilution point performed in 2-wells could be used). Follow-up samples must be tested in triplicate (two replicates are acceptable only in cases with insufficient DNA or failed technical criteria in third replicate).

To check for unspecific amplifications, nonspecific DNA controls (PB-MNC) should be run in 3 or 6 replicates (*see* **Note 1**) and a no template control (NTC) at least in duplicate, for each specific target quantification, respectively.

The specific oligonucleotide primers and probe, as selected based on available IG/TR targets and sensitivity testing, must be used (*see* **Note 2**).

1. Prepare the reaction mixture for each sample/well as follows:

2× ddPCR Supermix for Probes (no dUTP)	11.0 µl
20× target primers/probe mix	1.1 µl
(HINFI (2 U/µl) - optional)	*(1.1 µl)*
H_2O	4.4 µl
(volume must be modified if the enzyme is used)	
Total volume	**16.5 µl** (*see* **Note 3**)

2. Dispense the mix in the plate or in 0.2 ml strip tubes.
3. Add 5.5 µl of the DNA (100 ng/µl) sample for each well.
4. Seal the plate or the strips with optical adhesive film or caps, mix and spin down briefly.
5. Proceed with droplets generation (*see* **Note 4**).
   (a) Load 20 µl of reaction mix and 70 µl of droplet generation oil into the proper DG8 cartridge wells.

(b) Carefully remove any bubble created into the DG8 cartridge "sample" well during sample loading.

(c) Put the DG8 gasket and start the droplets generation.

6. Carefully transfer 40 μl of generated droplets into a ddPCR 96-well plate.
7. Seal with a pierceable foil on PX1 PCR plate sealer.
8. Start the amplification using the default Bio-Rad thermal cycling protocol (95 °C, 10 min; 94 °C, 30 s; proper Tm°C, 1 min for 40 cycles; 98 °C for 10 min) adjusting the Tm according to the ASO-primers annealing temperature.
9. Load the post-PCR 96-wells plate into the QX100/QX200 droplet reader and follow the manufacturer's instructions. Figure 1 shows a schematic diagram of a ddPCR experiment.

3.2 DNA Quantification Using the Reference Gene

A reference gene must be tested to correct the MRD value in the actual follow-up sample based on the quantity of DNA loaded. Although no consensus has been reached on reference gene usage, the albumin gene is the most frequently used housekeeping control gene. Details on primers and probe concentrations to amplify a portion of the albumin gene as a reference are indicated in **Note 2**. The reference gene is recommended to be tested (in a single well) in the same ddPCR plate as for the target gene.

1. Prepare the reaction mixture for each sample/well as follows:

2× ddPCR Supermix for Probes (no dUTP)	11.0 μl
20× target primers/probe mix	1.1 μl
(HINFI (2 U/μl) -optional)	[*1.1 μl*]
H2O	8.8 μl
(volume must be modified if the enzyme is used)	
Total volume	**20.9 μl** (*see* **Note 3**)

2. Dispense the mix in the plate or 0.2 ml strip tubes.
3. Add 1.1 μl of the DNA (100 ng/μl) sample in each well.
4. Seal the plate or the strips with optical adhesive film or caps, mix, and spin down briefly.
5. Proceed with droplets generation (*see* **Note 4**).

(a) Load 20 μl of reaction mix and 70 μl of droplet generation oil into the proper DG8 cartridge wells.

(b) Carefully remove any bubble created into the DG8 cartridge "sample" well during sample loading.

(c) Put the DG8 gasket and start the droplets generation.

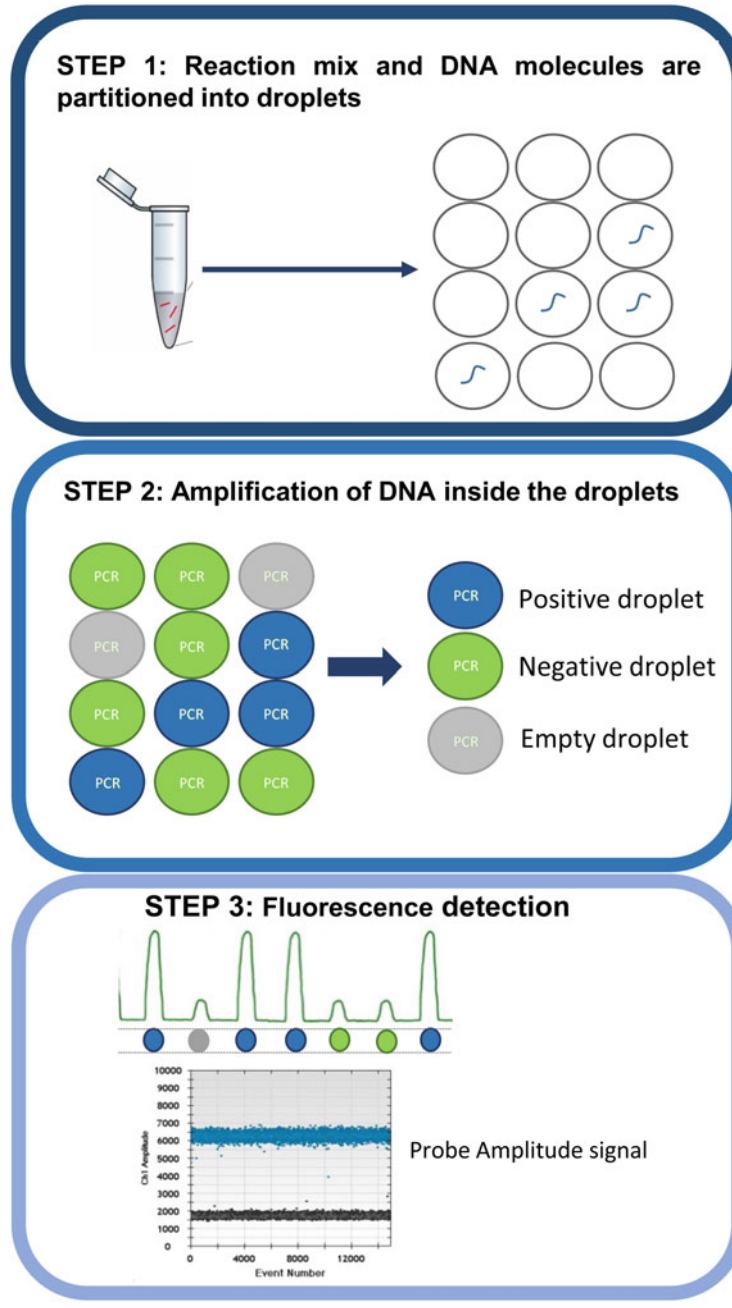

Fig. 1 ddPCR MRD quantification: schematic diagram of a ddPCR experiment. **Step 1**: the reaction mix is prepared with the same primer/probes as for the RQ-PCR assay. Both the reaction and the DNA samples are partitioned into 20,000 droplets of identical volume through a microfluidic system. **Step 2**: in a thermal cycler, 20,000 PCR reactions are amplified and fluorescence is the output during the reaction of polymerization. **Step 3**: a droplet reader analyzes each droplet individually and detects an increased fluorescence in positive droplets, which contain at least one copy of the target DNA

6. Carefully transfer 40 μl of generated droplets in ddPCR 96-well plates.
7. Seal with a pierce able foil on PX1 PCR plate sealer.
8. Start the amplification using the default Bio-Rad thermal cycling protocol (95 °C, 10 min; 94 °C, 30 s; annealing 60 °C, 1 min for 40 cycles; 98 °C for 10 min).
9. Load the post-PCR 96-wells plate in the QX100/QX200 droplet reader and follow the manufacturer's instructions.
10. At the end of the ddPCR reaction, analyze the plots. Check that no amplification is seen in the NTC and exclude samples with very low or high values, outside the range of 300–7500 copies/μl (corresponding theoretically to 20–500 ng) [21].

3.3 ddPCR Results Analysis

The analysis must be performed by QuantaSoft or QuantaSoft PRO according to the following criteria:

1. Only replicates with equal or more than 9000 droplets and equal or less than 20,000 must be considered for the analysis.
2. The threshold must be established manually. The threshold should be settled below the positive control cloud and as close as possible to the background signal (*see* **Note 5**).
3. Set a single threshold, based on the patient specific positive control, for all those samples that use the same set of ASO-primers. However, for those samples presenting unaligned amplitude signal (due to different background amplification signal related to the follow-up DNA quality), a sample specific threshold must be set.
4. In case of few positive events in follow-up samples, NTC or PB-MNC wells, verify the consistency of the amplification signal by checking for the presence of positive droplets in channel 2 (ch2). If a signal in ch2 is detected, in the same position of the ch1 signal, this represents an unspecific amplification (false-positive signal) and must be excluded from the analysis (Fig. 2).

3.4 Interpretation of ddPCR MRD Results

An excel sheet can be used to report all IG/TR target amplification values for all follow-up samples. In the process of setting an international standardization, ddPCR results have been interpreted so far with different guidelines [21, 22]. See Table 1 for the provisional EuroMRD guidelines.

Interpretations must be incorporated into the clinical report. Although it has not been standardized so far, just as for RQ-PCR, a clinical report ideally should contain the following information for each follow-up sample analyzed: date and type of sampling, the actual MRD value, and the corresponding quantitative limit (QL). If the MRD value is positive but below the quantitative limit, the

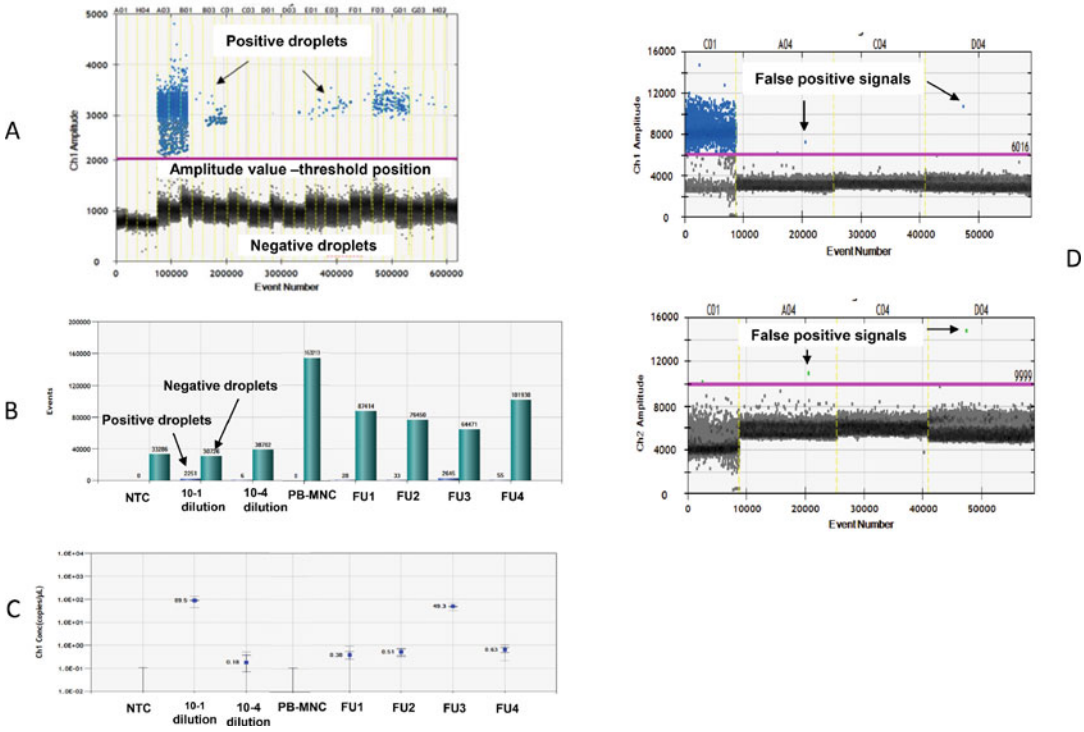

Fig. 2 ddPCR results analysis: each droplet is plotted on the graph of fluorescence intensity versus droplet number (**a**). The concentration is calculated on the fraction of empty droplets (green bar), which is the fraction that does not contain any target DNA (**b**). Fraction of positive droplets is fitted to a Poisson algorithm to determine the absolute copy number, and results are presented in copies per µL (**c**). In case of few positive events in follow-up samples, NTC or PB-MNC wells, verify the consistency of the amplification signal by checking for the presence of positive droplets in channel 2. If a signal in ch2 is detected, in the same position of ch1 signal, this represents an unspecific amplification (false-positive signal) and must be excluded from the analysis (**d**). (*Adapted from Della Starza I,* et al *Front Oncol. 2019 Aug 7;9:726*)

Table 1
Provisional EuroMRD guidelines for ddPCR

Data interpretation	Provisional EuroMRD guidelines [20]
MRD positive sample	A merge of events ≥3, regardless of number of positive replicates After verification for unspecific signals, in the presence of positive background, the calculation has to be: Copies MRD sample - copies background
MRD negative sample	All acceptable replicates with a merge of no or only one event
MRD positive below the quantitative limit	A merge of event = 2 After verification for unspecific signals, in the presence of positive background, the calculation has to be: Copies MRD sample - copies background
MRD quantification	Target copies in 500 ng of DNA are calculated as the mean values of replicates (mean copies/µl × 20 µl)

value can be reported as "POS < QL" (i.e., POS < 1.0×10^{-4}). As already established for RQ-PCR results, this qualitative result cannot be further interpreted: it only means that the sample is positive and lower than the QL, but it cannot be quantified precisely and should not be used for clinical decision, in particular not for upgrading the therapy, because of the intrinsic risk of false-positivity. In case of negative MRD, the actual QL and the specific time point need to be considered for clinical interpretation and decision-making.

3.5 Conclusion

During the last years, many publications have reported on the ddPCR application in different hematological diseases. Its intrinsic characteristics (accuracy, sensitivity, quantification without the need of a standard curve, etc.) make this method also attractive for MRD evaluation. However, at the moment, the use of ddPCR as a MRD molecular method in clinical protocols is prevented by the lack of published international guidelines for data interpretation, which is a fundamental requirement to ensure reproducibility and to compare MRD data in different clinical protocols. For this reason, a major standardization effort is underway within the EuroMRD consortium groups, and five ddPCR QC rounds have so far been performed, involving 24 laboratories around the world [21]. The further challenges will be to achieve this goal and to assess the prognostic relevance of ddPCR in large studies in the light of its future application in clinical practice.

4 Notes

1. Since ddPCR allows accurate quantification of rare events, we suggest to use the same number of replicates (=3) for patients and for PBMNC samples.
2. Primers and probe should be used at a final concentration of 500 nM and 200 nM, respectively. For 25 μl total volume of 20X target primers/probe mix: 1 μl probe (100 μM), 2.5 μl each primers (100 μM), and 19 μl H_2O. For ddPCR analysis, BHQ1 or MGB or Zen probes should be used. TAMRA probes must be avoided since they lead to high background and noise signals.
3. Prepare the ddPCR mix for a volume increased by 10% to be sure to have enough reaction mix volume for each replicate, and not to risk the generation of air bubbles into the DG8 cartridges, when loading the samples.
4. Based on the DNA extraction method, gDNA could be viscous and this characteristic can affect the droplets generation. In case of sticky DNA, add 2 U/μl of enzyme (1.1 μl) to the

ddPCR reaction mix, adjusting properly with water. Importantly, before using the enzyme, verify that target sequences or primers and probes will be not damaged.

5. In case of one or two positive droplets, in the PB-MNC wells, just above the background signal, threshold line could be settled just above these observed droplets of the PB-MNC. In case of positive droplets in the PB-MNC samples at higher amplitude respect to the cloud of positive control, these are unspecific signals and must be omitted from the analysis.

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