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Minimal Residual Disease Detection by Droplet Digital PCR in Multiple Myeloma, Mantle Cell Lymphoma, and Follicular Lymphoma

A Comparison with Real-Time PCR

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Real-time quantitative PCR (qPCR) is a well-established tool for minimal residual disease (MRD) detection in mature lymphoid malignancies. Despite remarkable sensitivity and specificity, qPCR has some limitations, particularly in the need for a reference standard curve, based on target serial dilutions. In this study, we established droplet digital PCR (ddPCR) for MRD monitoring in multiple myeloma, mantle cell lymphoma, and follicular lymphoma and compared it head-to-head with qPCR. We observed that ddPCR has sensitivity, accuracy, and reproducibility comparable with qPCR. We then compared the two approaches in 69 patients with a documented molecular marker at diagnosis (18 multiple myelomas, 21 mantle cell lymphomas assessed with the immunoglobulin gene rearrangement, and 30 follicular lymphomas with the use of the BCL2/immunoglobulin gene major breakpoint region rearrangement). ddPCR was successful in 100% of cases, whereas qPCR failed to provide a reliable standard curve in three patients. Overall, 222 of 225 samples were evaluable by both methods. The comparison highlighted a good concordance ($r = 0.94$, $P < 0.0001$) with 189 of 222 samples (85.1%; 95% CI, 80.4%—89.8%) being fully concordant. We found that ddPCR is a reliable tool for MRD detection with greater applicability and reduced labor intensiveness than qPCR. It will be necessary to authorize ddPCR as an outcome predictor tool in controlled clinical settings and multilaboratory standardization programs. *(J Mol Diagn 2015, 17: 652—660; http://dx.doi.org/10.1016/j.jmoldx.2015.05.007)*

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Detection of minimal residual disease (MRD) allowed acquisition of valuable prognostic information in several mature lymphoid malignancies with a considerable impact on clinical research.[1,2] Currently, it is often included as a secondary end point in clinical trials for multiple myeloma (MM), mantle cell lymphoma (MCL), and follicular lymphoma (FL).[3–5] More recently, several cooperative groups have designed MRD-based risk-adapted studies in a number of therapeutic settings.[6]

Different methods can be used for MRD quantification, including flow cytometry (FC),[7–9] real-time quantitative PCR (qPCR),[10–13] and the more recent next-generation sequencing (NGS).[14,15] So far, qPCR remains the most validated and standardized method in MCL and FL.[4,5] In MM, for which FC also has a major role,[16] the International Myeloma Working Group has included molecular complete response (tumor marker negativity by PCR at sensitivity 10^{-5}), as a meaningful criterion for response evaluation.[17] In MM and MCL, qPCR uses immunoglobulin gene (*IGH*) rearrangement as a clonal marker, whereas in FL the most reliable marker is the t(14;18) translocation, especially when the major breakpoint region (*BCL2/IGH MBR*) is involved.[18]

qPCR represents the most widely used method for MRD analysis. However, it has a major limitation from being a relative quantification approach. This results in the need of a reference standard curve usually built by dilutions of the tumor-specific target obtained from diagnostic DNA, plasmids, or cell lines that contain the rearrangement of interest. Moreover, qPCR is unable to provide reliable target quantification for a substantial proportion of samples that have a tumor burden between the sensitivity and the quantitative range of the method. Samples that fall in this window of inadequate quantification, which might range up to two logs and are sometimes difficult to categorize for clinical purposes, are usually defined as positive nonquantifiable (PNQ).[19]

Droplet digital PCR (ddPCR) is based on sample compartmentalization in single oil droplets that represent independent PCR reactions and on end point amplification and Poisson statistics.[20–24] ddPCR has several theoretical advantages compared with qPCR,[25–29] most notably allowing for absolute quantification of target DNA molecules and avoiding the need for a reference standard curve; thus, it is potentially valuable in the MRD setting.

On the basis of these considerations, we sought to verify the utility of ddPCR as a MRD monitoring tool and to compare it head-to-head with qPCR in 69 patients, including 18 with MM, 21 with MCL, and 30 with FL for a total of 225 samples. Our aim was to verify whether ddPCR could overcome some limitations of qPCR without losing its critical advantages, especially in terms of sensitivity and reproducibility.

Materials and Methods

Sample Characteristics and DNA Extraction

Preliminary evaluation of ddPCR performance was conducted with plasmid and purified neoplastic cell dilutions for the *IGH* rearrangement and the DOHH-2 cell line for the *BCL2/IGH MBR*, as previously reported.[10,30,31] For method comparison, genomic DNA (gDNA) derived from bone marrow (BM) and peripheral blood (PB) samples from 69 patients (18 with MM, 21 with MCL, and 30 with FL) was used. Samples were selected for having a molecular marker on the basis of the *IGH* (MM and MCL) or *BCL2/IGH MBR* (FL) rearrangements and were collected in the context of prospective clinical trials approved by the local institutional review board (MCL: EUdract2009-012807-25; MM: Eudract2004-000531-28 and Eudract2008-008599-15; FL: Eudract2009-012337-29). All patients provided written informed consent, which included PCR-based MRD determination, according to the Helsinki Declaration. Overall, 225 samples (180 BM and 45 PB) were analyzed: 95 MM, 70 MCL, and 60 FL. A total of 70 were diagnostic samples [for one patient two diagnostic samples (BM, PB) were available], and 155 were taken during patient follow-up on the basis of availability of DNA (Supplemental Table S1). MCL and FL sample mononuclear cells were separated by density gradient (Histopaque-1077; Sigma-Aldrich, St. Louis, MO), whereas MM samples were treated with erythrocyte lysis buffer. gDNA was extracted, depending on the amount of cells, by DNAzol (Life Technologies-Invitrogen, Carlsbad, CA) or NucleoSpin Tissue (Macherey-Nagel, Bethlehem, PA), according to the manufacturer's recommendations. gDNA quality and concentration were estimated by Nanodrop 2000C (Fisher Thermo Scientific, Waltham, MA) before experimental use. To avoid possible biases related to sampling, qPCR and ddPCR quantification were performed on the same diluted gDNA samples. Detailed information is included in Supplemental Table S2, as suggested by the guidelines for the Minimum Information for the Publication of Digital PCR Experiments (dMIQE).[32]

Tumor-Specific Molecular Marker Assessment

In MM and MCL, patient-specific *IGH* rearrangements were amplified and direct sequenced from diagnostic gDNA.[10,31] Sequences were analyzed with the IMGT/V-QUEST tool (*http://imgt.org/IMGT_vquest/share/textes*, last accessed March 26, 2015),[33,34] and patient-specific allele-specific oligonucleotide primers and consensus probes were designed as previously described.[10] FL patients were screened at diagnosis for the *BCL2/IGH MBR* translocation, as already described.[18]

qPCR

IGH-based and *BCL2/IGH* MBR-based MRD detection by qPCR was performed with an AbiPrism7900HT (Life Technologies-Applied Biosystems, Carlsbad, CA), as previously described.[18,19] For each patient, sample estimation was based on serial 10-fold dilution standard curves, prepared according to Euro-MRD guidelines, as previously

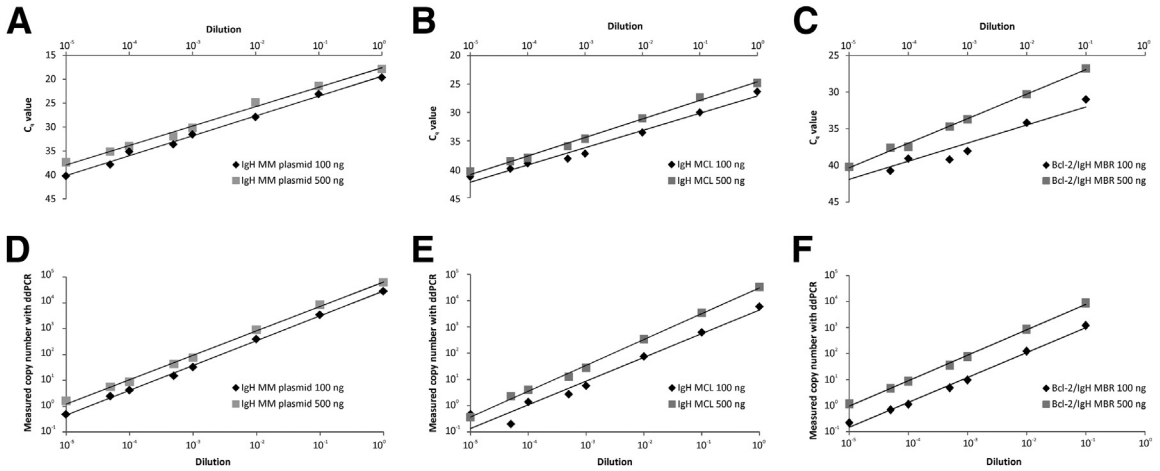

Figure 1 Comparison of serial DNA dilutions assessed by both qPCR and ddPCR. Linear 10-fold dilution standard curve with the Cq value for qPCR (**A**–**C**) and copy numbers for ddPCR (**D**–**F**) were plotted against the corresponding starting quantity of gDNA. **A** and **D:** Plasmid standard curves from an IGH rearrangement derived from a MM patient; 500 ng (R^2 = 0.9934) versus 100 ng (R^2 = 0.9951) by qPCR (**A**) and 500 ng (R^2 = 0.9978) versus 100 ng (R^2 = 0.9986) by ddPCR (**D**). **B** and **E:** gDNA standard curves from a MCL patient-specific *IGH* rearrangement; 500 ng (R^2 = 0.9948) versus 100 ng (R^2 = 0.9796) by qPCR (**B**) and 500 ng (R^2 = 0.9979) versus 100 ng (R^2 = 0.9640) by ddPCR (**E**). **C** and **F:** gDNA from Bcl-2/IGH MBR$^+$ cell line (DOHH-2); 500 ng (R^2 = 0.9966) versus 100 ng (R^2 = 0.9016) by qPCR (**C**) and 500 ng (R^2 = 0.9980) versus 100 ng (R^2 = 0.9937) by ddPCR (**F**). R^2 (coefficient of determination), represents how well the experimental data fit the regression line. Each dilution point represents the mean value of quantifiable replicates for different template amount (500 ng and 100 ng). ddPCR, droplet digital PCR; gDNA, genomic DNA; MBR, major breakpoint region; MCL, mantle cell lymphoma; MM, multiple myeloma; qPCR, quantitative real-time PCR.

described,[19] starting from i) 10^5 plasmids that contain the patient-specific *IGH* rearrangement for MM; ii) 500 ng of diagnostic gDNA derived either from unpurified or CD19$^+$ purified cells for MCL; and iii) 500 ng of DOHH-2 (BCL2/IGH MBR$^+$ cell line) gDNA, diluted in MCF-7 (BCL2/IGH MBR$^-$, human breast cancer cell line) gDNA for FL. In MCL, the proportion of tumor cells in diagnostic samples was assessed by standardized four-color FC for CD19, CD5, and κ/λ light chains (Miltenyi Biotec, Bergisch Gladbach, Germany)[7]; MRD analysis was interpreted according to the Euro-MRD guidelines.[19]

ddPCR

ddPCR was performed with the QX100 Droplet Digital PCR system (Bio-Rad Laboratories, Hercules, CA). gDNA samples were loaded in triplicate with the use of either the manufacturer-recommended 100 ng gDNA dose, or an increased amount of 500 ng (aiming at greater sensitivity). The 20 μL ddPCR reaction included 10 μL of 2× ddPCR Master Mix (Bio-Rad Laboratories), 1 μL of 20× primers and probe (final concentration, 500 nmol/L and 200 nmol/L), and 5 μL of gDNA. Of note, ddPCR experiments used

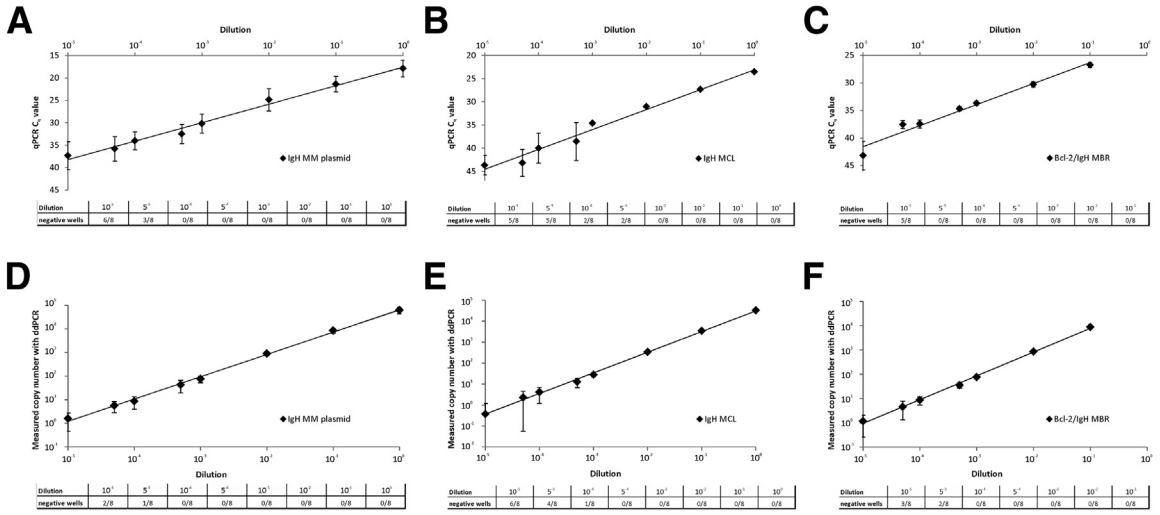

Figure 2 Sensitivity and accuracy of qPCR and ddPCR on serial DNA dilution. **A**–**C:** qPCR data. **D**–**F:** ddPCR data of 500 ng DNA standard curves. Each dot represents a mean value of eight replicates, with SD shown as whiskers. Table below each graph shows the amount of negative replicates at each dilution point. ddPCR, droplet digital PCR; MBR, major breakpoint region; MCL, mantle cell lymphoma; MM, multiple myeloma; qPCR, quantitative real-time PCR.

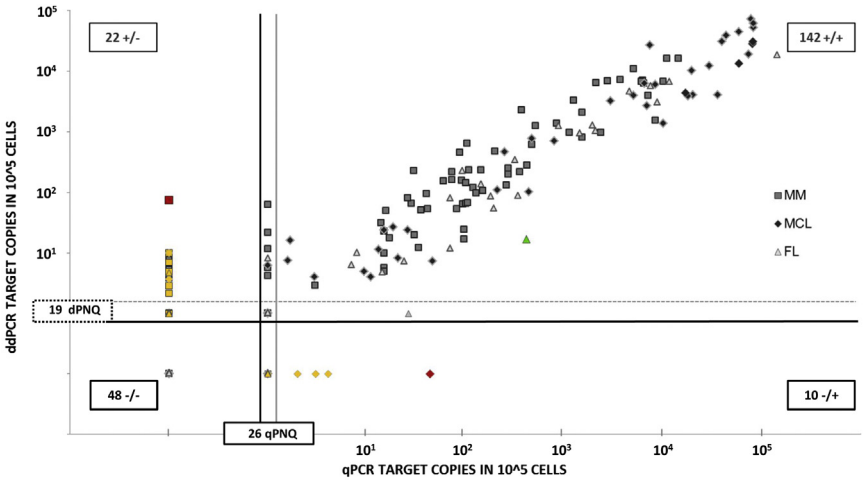

Figure 3 Correlation between qPCR and ddPCR in patient samples. Pearson correlation between target quantification in MM, MCL, and FL by both methods shows a significant concordance ($r = 0.94$, $P < 0.0001$). Forty-eight samples were undetectable by both methods (−/−). One hundred forty-two samples were scored positive by both methods, 141 of which were fully concordantly positive and 1 quantitative discordance (green). Major and minor qualitative discordances are labeled in red and yellow, respectively. PNQ samples are included between lines. Thirteen cases scored PNQ by both methods. ddPCR, droplet digital PCR; dPNQ, PNQ determined by ddPCR; FL, follicular lymphoma; MCL, mantle cell lymphoma; MM, multiple myeloma; PNQ, positive nonquantifiable; qPNQ, PNQ determined by qPCR; qPCR, quantitative real-time PCR.

the same primers and probes used in qPCR, with the identical nucleotide sequence, although MGB or BHQ-1 quenchers were used instead of TAMRA (Primmbiotech, West Roxbury, MA). Droplets were generated by a QX100 droplet generator device, and end point PCR was performed on a T100 Thermal Cycler (Bio-Rad Laboratories) following the manufacturer's recommendations. PCR products were loaded into the QX100 droplet reader and analyzed by QuantaSoft version 1.2 (Bio-Rad Laboratories). Each experiment included a positive control sample (diagnostic sample gDNA) and a negative control (pool of PB mononuclear cells from 10 healthy donors, or MCF-7 gDNA). The final tumor load was calculated as a mean of all available technical replicates that were considered reliable when giving reproducible amplification after application of a Poisson correction. To be consistent with the rules established by Euro-MRD for qPCR, we defined MRD^+ by ddPCR as those samples that had at least one replicate equal or superior to 1 log (equivalent to three Cq by qPCR) to the highest background signal. Finally, on the basis of the higher variability observed at low target concentrations and applying the EURO-MRD guidelines, cases with alternatively positive or negative replicates were scored as PNQ (PNQ determined by ddPCR, dPNQ).[19]

Sensitivity, Accuracy, and Reproducibility

To assess sensitivity, accuracy, and reproducibility of ddPCR in comparison with qPCR, serial 10-fold dilutions were performed with plasmids, the DOHH-2 cell line, and

Table 1 Discordances Observed between qPCR and ddPCR

Type of discordance	MM ($n = 95$)	MCL ($n = 67$)	FL ($n = 60$)	Total ($N = 222$ samples)
Major qualitative discordances, *n* (%) ($MRD^+ > 1 \times 10^{-4}$)	1 (1.1)	1 (1.5)		2 (0.9)
ddPCR positive	1 (1.1)			1 (0.45)
qPCR positive		1 (1.5)		1 (0.45)
Minor qualitative discordances, *n* (%) ($MRD^+ \leq 1 \times 10^{-4}$)	17 (17.9) (2 qPNQ-2 dPNQ)	7 (10.4) (2 qPNQ)	6 (10.0) (2 qPNQ-3 dPNQ)	30 (13.5) (6 qPNQ-5 dPNQ)
ddPCR positive	15 (15.8)	2 (3.0)	4 (6.7)	21 (9.5)
qPCR positive	2 (2.1)	5 (7.5)	2 (3.3)	9 (4.1)
Quantitative discordances, *n* (%) (discrepancy ≥1 log)			1 (1.7)	1 (0.45)
ddPCR higher				
qPCR higher			1 (1.7)	1 (0.45)
Total, *n* (%)	18 (19)	8 (11.9)	7 (11.7)	33 (14.9)
ddPCR positive or higher	16 (16.8)	2 (3.0)	4 (6.7)	22 (9.9)
qPCR positive or higher	2 (2.1)	6 (8.9)	3 (5.0)	11 (4.9)

Numbers in the table refer to the total number of comparable samples, sorted by disease. Amount and type of discordances recorded for each disease are summarized. Definitions of discordances are reported in the manuscript in *Materials and Methods*.

ddPCR, droplet digital PCR; dPNQ, PNQ determined by ddPCR; FL, follicular lymphoma; MCL, mantle cell lymphoma; MM, multiple myeloma; MRD, minimal residual disease; PNQ, positive nonquantifiable; qPNQ, PNQ determined by qPCR; qPCR, quantitative real-time PCR.

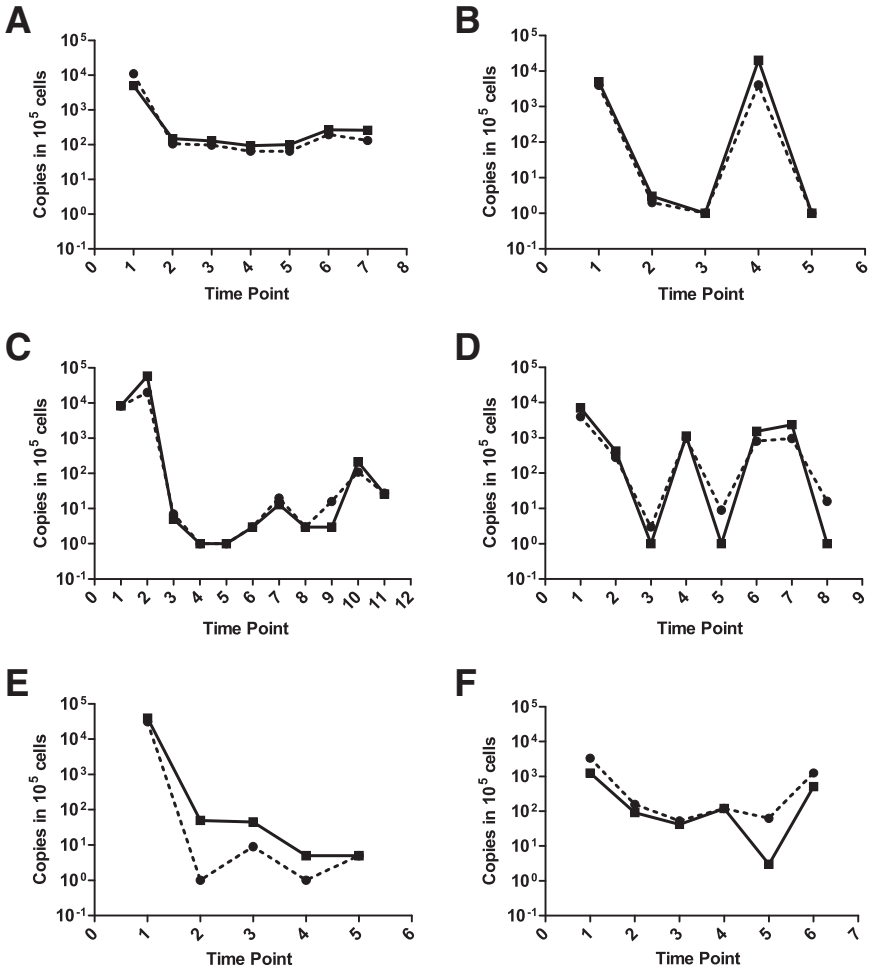

Figure 4 Example of MRD detection discordances in patient's follow-up samples. Comparison of MRD level at different tps, taken on the basis of availability of DNA, evaluated by qPCR (solid lines) and ddPCR (dashed lines), for three MM (**A**, **D**, and **F**) and three MCL (**B**, **C**, and **E**) patients. **A**–**C:** Three patients with fully concordant follow-up samples. **D:** A patient with three minor qualitative discordances (tp 3, 5, and 8). **E:** One case with a major qualitative discordance (tp 2) and a minor qualitative discordance (tp 4). **F:** A follow-up sample PNQ by qPCR but quantifiable by ddPCR at tp 5. The amount of target copies was $\log_{10}$ transformed. ddPCR, droplet digital PCR; MCL, mantle cell lymphoma; MM, multiple myeloma; MRD, minimal residual disease; PNQ, positive nonquantifiable; qPCR, quantitative real-time PCR; tp, time point.

purified tumor cells. We tested from 10^5 copies to 10^0 copy (including twofold intermediate dilution steps 5×10^{-4} and 5×10^{-5}) of *IGH* target, diluted in gDNA obtained from a pool of PB mononuclear cells from 10 healthy donors and from 10^4 copies to 10^0 copy of *BCL2/IGH MBR* target diluted in MCF-7 gDNA. These dilutions were escalated to eight replicates to verify accuracy and reproducibility at different dilution steps. Standard curves quantification was tested by both ddPCR and qPCR and repeated over time to assess whether the consistency (therefore, the data accuracy and reproducibility for the samples) was maintained.

Analysis of Discordances

Discordances were classified as previously described.[14] Discordances in terms of positivity versus negativity were defined as qualitative discordances and were classified as major qualitative discordance, when a positive result was $>1 \times 10^{-4}$, or minor qualitative discordance, when the positive result was $\leq 1 \times 10^{-4}$. Of note in this group, discordances might be related to statistical variability or minimal differences in sensitivity. Furthermore, concordantly positive results, but with quantitative discrepancy >1 log, were defined as quantitative discordances.

Statistical Analysis

For methods comparison, qPCR and ddPCR results were expressed as the amount of target copies per 10^5 cells. qPCR and ddPCR comparability was assessed with bivariate Pearson correlation between methods calculated as an index of inter-method reliability of quantitative data (R version 3.1.0, package irr; R Foundation for Statistical Computing, Vienna, Austria). The variance of ratings did not differ between methods. The strength of agreement between the two methods was also calculated with the Bland-Altman difference analysis with a 95% limit of agreement (Supplemental Figure S1).[35]

Results

Sensitivity, Accuracy, and Reproducibility of ddPCR over Different Targets and Tumor Levels

To assess the sensitivity of ddPCR in tumor target detection, we performed a series of 10-fold dilutions, loading both 100 ng of gDNA, as recommended by general ddPCR protocols, and 500 ng, which is the amount of gDNA usually used in the MRD setting.[19] Representative results for *IGH* (with the use of the patient-specific *IGH* rearrangement incorporated

into a plasmid from a MM patient and tumor cells taken at diagnosis from a heavily infiltrated MCL patient) and *BCL2/IGH MBR* (cell line) target quantification are shown in Figure 1, A–C, and compared with qPCR (Figure 1, D–F). Of note, scaling up the ddPCR reaction to 500 ng did not have a negative impact on reaction performance, as already described in other studies.[36] ddPCR ensured quantitative discrimination over a broad range of target amounts (10^5 to 10^0), which were comparable with qPCR also at low levels of target concentration. Moreover, the use of 500 ng of gDNA (ie, 10^5 copies at the first dilution step) allowed reaching a sensitivity of 10^{-5} in all settings. As theoretically expected, the use of 100 ng prevented amplification of the lowest dilution in some cases.

We then examined the concordance between ddPCR and qPCR on serial dilutions. Our results found the excellent concordance of the two methods at different levels of target concentration (Supplemental Figure S2, A–C). Then we compared the reproducibility of the two methods by performing ddPCR and qPCR on eight replicates for each of the three types of dilutions (in at least two separate experiments for every target). Again, the two methods were highly comparable in terms of reproducibility with minimal divergence between replicates by both tools. As expected, with both methods, the 10^{-5} dilutions scored some negative replicates, reflecting Poisson statistics (Figure 2).

Feasibility of MRD Detection by the Two Approaches

Overall, MRD analysis was successful in 95.6% of patients (66 of 69) by qPCR and 100% by ddPCR. In total, 100% of MM and FL samples were evaluated by both methods. In contrast, in three MCL patients, qPCR failed to generate reliable results because of the production of an inadequate standard curve, according to Euro-MRD guidelines, whereas ddPCR performed successfully.[19] Of note, two of these three diagnostic samples were analyzed by FC and indicated high tumor infiltration (60%, 79% of $CD5^+$/$CD19^+$ cells), indicating that quantification failure was not caused by minimal infiltration, and one indicated a low amount of tumor cells by FC. Because qPCR was unsuccessful in three cases, method comparison was used in 67 diagnostic samples from 66 patients (for one patient both BM and PB at diagnosis were analyzed) and 155 follow-up samples (Supplemental Table S1).

Concordance Analysis

A total of 222 of 225 samples (98.7%) were evaluated by both methods and included in the concordance analysis. As expected, when the *BCL2/IGH* was used, we never observed positivity in the no-template samples, whereas in the *IGH* rearrangement setting we observed a background amplification signal in 4 of 39 patients (10.3%) by ddPCR (consisting of one or two events in only one of the replicates) and in 3 of 36 (8.3%) by qPCR. Of note, in two cases the nonspecific background was detected by both tools overlapped. In total, we evaluated 67 diagnostic and 155 follow-up samples (Supplemental Table S1) that were quantifiable on the basis of a standard curve built with plasmids (for MM), purified cells (for MCL), or a BCL2/IGH MBR^+ cell line (for FL). Of these, 95 samples were MM (18 diagnostic BM and 77 follow-up), 67 were MCL (18 BM and 4 PB diagnostic and 45 follow-up samples), and 60 were FL (27 diagnostic and 33 follow-up samples). A highly significant level of concordance was observed between qPCR and ddPCR ($r = 0.94$, $P < 0.0001$; 95% CI, 0.9495–0.9712) (Figure 3 and Supplemental Figure S2). Of 22 samples, 189 (85.1%; 95% CI, 80.4%–89.8%) were fully concordant. MRD detection was fully concordantly positive in 141 of 222 samples (63.5%; 95% CI, 57.2%–69.8%; 75 MM, 41 MCL, and 25 FL) and concordantly negative in 48 of 222 (21.6%; 95% CI, 16.2%–27%; 8 MM, 11 MCL, and 29 FL), whereas 33 of 222 samples (14.9%; 95% CI, 10.2%–19.6%) were identified as discordant.

Discordances between qPCR and ddPCR quantification are reported in Table 1. On the basis of the previously reported criteria for discordance, only 2 of 222 samples (0.9%; 1 MM, 1 MCL) exhibited a major qualitative discordance (*Analysis of Discordances*). Of interest, in the MM case, qPCR found a nonoptimal sensitivity of 10^{-4}. Most cases, 30 of 222 (13.5%; 17 MM, 7 MCL, and 6 FL), were indeed classified as minor qualitative discordances, which might reflect Poisson statistic discrepancies related to the low number ($<10^{-4}$) of tumor cells in the sample. In 1 of 222 cases (0.45%), a quantitative discordance was observed to occur in a FL sample (Figure 3).

All of the discordances occurred in follow-up samples and did not appear to cluster in specific patients, with the exception of two MM patients: one showing three minor discordances (Figure 4D) and one displaying four qualitative discordances (one major, three minors) (data not shown). Of note, 22 of 222 samples (9.9%) were scored positive or higher by ddPCR (median, 7 copies; range, 2 to 74 copies), whereas 11 of 222 samples (4.9%) were scored positive or higher by qPCR (median, 13 copies; range, 2 to 44 copies). Interestingly, among minor discordances, an excess of positive cases was observed by ddPCR compared with qPCR, mostly in MM cases (Table 1). Figure 4 shows representative examples of MRD levels assessed by both methods, including three fully concordant cases (two MCL and one MM) (Figure 4, A–C), two cases with minor qualitative discordances (one MM and one MCL) (Figure 4, D and E, respectively), and one case with a major qualitative discordance (Figure 4E). Of note, one MM patient showed a follow-up sample PNQ by qPCR (qPNQ) but quantifiable by ddPCR (Figure 4F).

PNQ Samples

On the basis of Euro-MRD guidelines,[19] 26 of 222 samples (11.7%) were qPNQ. According to our definition, 19 of 222 samples (8.6%) scored dPNQ. Of 222 samples, 13 (5.9%) were PNQ by both methods. For qPNQ cases, in 7 of 26

samples (26.9%; five MM, one MCL, and one FL) ddPCR was able to provide a reliable quantitative result (median, 8 copies; range; 6 to 63 copies), whereas in 6 of 26 cases (23.1%; two MM, two MCL, and two FL) no amplification signal was observed, and samples were scored negative by ddPCR. In the dPNQ group, 5 of 19 samples (26.3%) scored negative by qPCR, whereas only 1 of 19 (5.3%) was quantifiable by qPCR.

Discussion

In this study, we used for the first time ddPCR in the context of MRD evaluation in MM, MCL, and FL patients and compared its performance with the well-established qPCR-based method. Our results indicate that i) ddPCR has sensitivity, accuracy, and reproducibility at least comparable with qPCR; ii) ddPCR allows bypassing the development of a dilution-based standard curve with substantial benefit in terms of reduced costs and labor intensiveness; and iii) ddPCR and qPCR exhibited excellent correlation in all of the assessed disease entities and over a broad range of tumor infiltration rates. On the basis of these findings, ddPCR may be considered an attractive alternative to qPCR for MRD evaluation in mature B-cell lymphoid malignancies.

Our data indicate that ddPCR is at least superimposable on qPCR. Nevertheless, ddPCR has several practical advantages, mostly related to its absolute quantification nature with no need for a standard curve and easier data interpretation. This ensures quantification of samples in which a standard curve could not be built and results in reduced cost, labor intensiveness, and spares precious diagnostic tissues, as summarized in Supplemental Table S3. Of note, the starting point for building the qPCR standard curve consists either of unpurified diagnostic tissue (needing FC quantification of tumor cell invasion not always superimposable on molecular results) or purified cells or plasmids, which are both expensive and laborious to generate. In addition, ddPCR ensures direct homogeneous absolute quantification of diagnostic and follow-up samples. Moreover, ddPCR is less susceptible to potential PCR performance inhibitors, such as anticoagulants, DNA extraction residual reagents (alcohol), or blood components (heme) and should allow easier multiplexing set-up.[37–42]

One relevant issue was to verify that ddPCR might reach a sensitivity that is comparable with qPCR. Recommendations from the producer indicate that the optimal performance of ddPCR is expected to occur when 100 ng of gDNA is used in each reaction. This would represent a major limitation for MRD purposes because achieving optimal sensitivity requires larger DNA amounts. However, our results clearly show that ddPCR also has excellent MRD performance when, as recommended by Euro-MRD guidelines, 500 ng of gDNA is used.[19] This result, which mirrors similar observations in unrelated disorders,[27,36] allowed us to reach sensitivity levels comparable with qPCR and perfectly suitable for MRD purposes.

The rate of concordance between qPCR and ddPCR is indeed high and superior to what we observed in a recent comparison between qPCR and NGS-based MRD analysis. Notably, most discordances were observed in the presence of a low number of target copies (minor qualitative discordances), which might reflect variability associated to Poisson's statistics and subtle differences in sensitivity usually in favor of ddPCR.

One critical limitation of qPCR is the frequent occurrence of cases that are defined as PNQ, which reflects the often-observed gap between sensitivity and the quantitative range, resulting in cases for which a truly quantitative value cannot be defined. Because ddPCR does not require a standard curve, on the basis of reproducibility data and good comparison with qPCR, whenever ddPCR is positive in all replicates, the results can be considered as positive and quantifiable. However, we decided to maintain the PNQ definition also for ddPCR for cases in which only a fraction of replicates was PCR^{+}, because a much larger number of replicates would be necessary for a reliable quantification of these samples.

One important achievement in the qPCR field was the multilaboratory standardization established by large cooperative efforts such as the Euro-MRD group. Indeed, ddPCR still has to undergo such a critical validation step. However, considering the reduced labor intensiveness of the experimental set-up and the current robust structure of multilaboratory validation groups, we believe that ddPCR standardization would be definitely easier and faster than qPCR. Another important issue could be the application of ddPCR to the broad range of different targets that are used in acute lymphoblastic leukemia.[43]

Recently, our group and others have shown the value of NGS in the MRD setting.[14,15,44] We think that NGS might substitute for PCR-based MRD tools in a number of settings. However, NGS has a substantial intrinsic complexity and involves major costs. Furthermore, robust and broadly applicable NGS-based MRD protocols are still not available in academic laboratories; moreover, its superiority in comparison with qPCR has not been proved in the context of all diseases. Although NGS ensures a higher rate of marker identification in MM, results are broadly superimposable in MCL, whereas reliability and cost-effectiveness are probably inferior in FL.[14,15,44] Therefore, we believe that PCR-based approaches will remain extensively used at least for the next 5 years. As a practical example, the new large phase 3 trials of the European MCL Network (Munich, Bavaria, Germany) will still rely on PCR for MRD monitoring.

Conclusion

ddPCR appears to be a feasible and attractive alternative method for MRD assessment and may complement or even substitute for qPCR in routine clinical laboratories. However, the potential advantages and predictive value need to

be further studied in the context of prospective clinical trials. On the basis of the results reported here, we are currently assessing MRD by both ddPCR and qPCR on four large prospective clinical trials (>800 patients) in the context of Fondazione Italiana Linfomi studies.

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Supplemental Data

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