

Digital PCR for *BCR-ABL1* Quantification in CML: Current Applications in Clinical Practice

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Abstract

Molecular monitoring of the *BCR-ABL1* transcript for patients with chronic phase chronic myeloid leukemia (CML) has become increasingly demanding. Real-time quantitative PCR (qPCR) is the routinely used method, but has limitations in quantification accuracy due to its inherent technical variation. Treatment recommendations rely on specific *BCR-ABL1* values set at timed response milestones, making precise measurement of *BCR-ABL1* a requisite. Furthermore, the sensitivity of qPCR may be insufficient to reliably quantify low levels of residual *BCR-ABL1* in patients in deep molecular response (DMR) who could qualify for an attempt to discontinue Tyrosine Kinase Inhibitor (TKI) therapy. We reviewed the current use of digital PCR (dPCR) as a promising alternative for response monitoring in CML. dPCR offers an absolute *BCR-ABL1* quantification at various disease levels with remarkable precision and a clinical sensitivity reaching down to at least MR5.0. Moreover, dPCR has been validated in multiple studies as prognostic marker for successful TKI treatment discontinuation, while this could not be achieved using classical qPCR. dPCR may thus prospectively be the preferred method to reliably identify patients achieving treatment milestones after initiation of TKI therapy as well as for the selection and timing for TKI discontinuation.

Introduction

Since the advent of targeted therapy with tyrosine kinase inhibitors (TKI), the landscape of chronic myeloid leukemia (CML) changed drastically with markedly improved prognosis and many patients achieving deep molecular remissions. The role of molecular techniques to quantify the *BCR-ABL1* transcript has become increasingly important, while the use of cytogenetic analyses diminished, as these are less sensitive and insufficiently informative.¹ Current European Leukemia Net (ELN) recommendations regarding response monitoring after first or second line TKI treatment rely primarily on the molecular response (MR), setting milestones at 3, 6 and 12 months based on well-defined *BCR-ABL1* values with or without additional cytogenetics.² As TKI therapy does not fully eradicate the leukemic clone, it was initially thought that treatment would need to be continued indefinitely. However, in recent years, the possibility of treatment discontinuation has been introduced for CML patients. Indeed, approximately 50% of CML patients in stable deep

molecular response (DMR) could safely cease TKI therapy without molecular relapse both in clinical trials and in real-world populations, entering a so-called treatment free remission (TFR).^{3–5} ELN recommendations regarding the selection of candidates for a TKI discontinuation attempt also depend on accurate molecular monitoring of minimal residual disease (MRD). Noteworthy, an attempt can be considered in both patients with detectable and undetectable disease, as long as the *BCR-ABL1* level is $\leq 0.01\%$ on the International Scale (IS). In fact, there was no difference in success rate between patients with detectable versus undetectable *BCR-ABL1* if quantified using conventional real-time quantitative PCR (qPCR).⁵ Interestingly, treatment discontinuation is even possible for at least some patients with *BCR-ABL1* levels above this cutoff of 0.01%IS.⁶

The polymerase chain reaction (PCR) to amplify a molecule was first described in 1983 and since then several new techniques developed, including qPCR and more recently digital PCR (dPCR). While conventional PCR is a qualitative end-point measurement of an amplicon (ie, present or not), qPCR enables quantification of the amplified product. The amount of target sequence in a qPCR reaction is calculated relative to a reference gene and can be quantified by measuring the patient's sample against a standard curve generated from serial dilutions with fixed copy numbers of (c)DNA. Currently, qPCR can detect one CML-cell in up to 100.000 normal cells. Differences in amplification efficiencies are in principle corrected for by standard curves. However, standard curves will not correct, for example, inhibitors in samples or inhibition due to a cDNA overloading. Therefore, a substantial variability is still observed in *BCR-ABL1* qPCR assays.⁷ This is especially the case at lower *BCR-ABL1* copy numbers due to differences in sensitivity of the

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assays and quality as well as quantity of template added, but even at higher copy numbers as a result of inherent methodological variability between diverse qPCR systems using different techniques and various control genes (eg, *ABL1*, *GUSB*, *BCR*).⁸ Consequently, world-wide assay harmonization has been the focus for years, however in the meantime a standardization in reporting *BCR-ABL1* results was necessary to ensure comparable results between laboratories.^{9–11}

This effort was undertaken by the three laboratories performing molecular analyses for the IRIS trial.^{12,13} For this trial, qPCR methods were aligned by determining a baseline *BCR-ABL1* value using the median value of 30 samples collected from newly diagnosed CML patients before any treatment was started and assayed in all three laboratories. A major molecular response (MMR) was defined as a 3-log reduction from this value. In the trial, the achievement of MMR was found to correlate with an excellent progression-free survival. Subsequently, a common reporting scale, the International Scale (IS), was introduced with MMR defined as 0.1%IS. The IS was designed to be independent of analysis platforms and selection of reference genes, by calibration and use of a conversion factor (CF). To express measured *BCR-ABL1* values on the IS, each CML-center could exchange samples with reference laboratories to determine their laboratory-specific CF or could use standardized kits and reagents calibrated by the World Health Organization (WHO) International Genetic Reference Panel for the quantification of *BCR-ABL1*.¹⁴ The implementation of the IS not only facilitated the comparison of results but also the execution of multicenter clinical trials where conformity of molecular results is much needed.

With the advent of more potent second and third generation TKI (2G and 3GTKI), an increasing proportion of CML patients were achieving molecular responses even deeper than MMR with sometimes undetectable disease levels. Therefore, an accurate and standardized definition of DMR according to the sensitivity of the *BCR-ABL1* quantification was proposed (and included in the ELN recommendations) as follows: MR4 either detectable disease $\leq 0.01\%$ IS *BCR-ABL1* or undetectable disease in cDNA with ≥ 10.000 *ABL1* transcripts; MR4.5 either detectable disease $\leq 0.0032\%$ IS *BCR-ABL1* or undetectable disease within cDNA with ≥ 32.000 *ABL1* transcripts; and MR5 either detectable disease $\leq 0.001\%$ IS *BCR-ABL1* or undetectable disease in cDNA with ≥ 100.000 *ABL1* transcripts.^{2,15,16}

Despite the efforts of standardization and the progress made to improve the qPCR technique (eg, the development of international standardized protocols, replicate qPCR),⁹ there is still a need for a more precise and more sensitive molecular assay. Since the ELN defined timed molecular milestones at specific *BCR-ABL1* target values, technical variation may result in misinterpretation of presumed biological variations and misclassification of treatment response. Although this has been in part accommodated by recommendations on repeated testing and the introduction of “warning” categories for the molecular targets, it remains a clinical pitfall. Failure to achieve these molecular quantitative targets has direct clinical consequences and may be an important reason for switching TKI therapy, even when a complete hematological response is achieved. For example, the recommended management of a CML patient below or above 1%IS at 6 months is substantially different: one can measure a *BCR-ABL1* value of 1,4%IS while the actual value would be 0,8%IS. Various observations were made that failure to achieve early molecular response milestones is associated with inferior survival and should perhaps trigger an early TKI

switch.^{17–19} In addition, with the novel goal of TFR and the emergence of 2GTKI deepening residual disease levels even more, it is crucial to obtain reliable and precise quantifications with minimized variation in single measurements for well-founded decision-making. In this review we focused on the current knowledge of *BCR-ABL1* dPCR for 1) monitoring of response to TKI initiation according to the ELN milestones, 2) for MRD detection and 3) as prognostic marker for the selection of candidates for a TKI discontinuation attempt.

Digital PCR

In classic qPCR an analogue measurement is performed during the exponential phase of the amplification. Subsequently, a threshold is set, the crossing point (Cp) or cycle quantification (Cq) value is determined and via standard curves converted into a copy number. dPCR however, generates a linear digital signal and can alleviate some shortcomings of qPCR as follows: (1) by partitioning the sample into thousands of independent PCR-reaction chambers the target is enriched, that is, increasing the effective concentration, thereby improving the detection limit; (2) by measuring the endpoint of amplification the assay is less prone to variation due to differences in amplification efficiency; (3) it bypasses the need of a calibrated standard curve and therefore enables absolute quantification of the target molecule with high precision and reproducibility.²⁰ Hence, it is used in an increasing number of quantitative applications such as the measurement of copy number variation in cancer, fetal karyotyping and the quantification of low-abundance sequences (eg, *BCR-ABL1* in CML).²¹ The main similarities and differences between dPCR and qPCR are listed in Table 1.^{20,22}

Principle of digital PCR

Various groups have contributed to improve the basic PCR technique to “digital” quantification.²³ Vogelstein and Kinzler coined the term in 1999, using this technology to detect a rare mutation in a large cell population.²⁴ In dPCR, single sequences are isolated in microreaction chambers or partitions, and individually amplified by PCR. By using fluorescent probes, the presence of the targets will result in a color change in the reaction chamber during the PCR amplification, determined in every separate partition as endpoint measurement which can be plotted on a one-dimensional scatter graph (see Fig. 1). With a well-optimized assay 2 separate populations can be distinguished, which results in a digital or binary readout. Since the fluorescence signal is still analogue, some partitions can fall in between the negative and positive group creating a “rain effect”. This effect is normally minimal and mainly caused by variation in amplification efficiency in each separate partition or by fragmented target DNA. However, as long as a positive partition exceeds the threshold, this variation does not have repercussions on the final result. In order to quantify the amount of target sequence in the reaction, a Poisson correction to the fraction of positive partitions is applied using the average occupancy per partition to compensate for partitions containing more than one sequence. Interestingly, if the average occupancy per partition is > 1 , the dynamic range of the assay (the highest number of target sequences detected) can extend beyond the number of partitions analyzed by more than a factor five. The Poisson distribution requires a large number of partitions of a fixed volume, an event with a binary outcome and a random distribution of molecules.

Table 1 A Comparison of Real-time Quantitative PCR and Digital PCR in General and in the Context of CML for BCR-ABL1 Quantification.		
	Real-time Quantitative PCR	Digital PCR
Similarities	Similar amplification reagents and fluorescent labeling Assay performance dependent on proper design Assay prone to sampling error Automated cartridge-based assays available Quantification of control gene for quality control and expressing on IS	
Differences	Relative quantification to standard curve Real-time detection Sensitivity and precision influenced by variation in amplification efficiency Clinical detection limit of MR4.0-MR5.0 Low inter-assay reproducibility Variable accuracy in detection of MRD ranging from a 1.2 to 8-fold bias	Absolute quantification, no need of a standard curve End-point detection Sensitivity and precision less influenced by variation in amplification efficiency Clinical detection limit of MR5.0-MR6.0 High inter-assay reproducibility Accurate detection of MRD with a 1.2-fold bias

FPR = false positivity rate; IS = international scale; MR = molecular response; MRD = minimal residual disease.

These characteristics are crucial to obtain a reliable quantification and are therefore the foundations of dPCR. For more information about the principles and statistics of dPCR in general, we refer to the review of Basu.²⁰

Sensitivity and specificity of the assay

The lower limit of detection (LoD) is a marker of analytical sensitivity and defined as the concentration of a target sequence detectable with 95% certainty. In dPCR, the LoD corresponds to

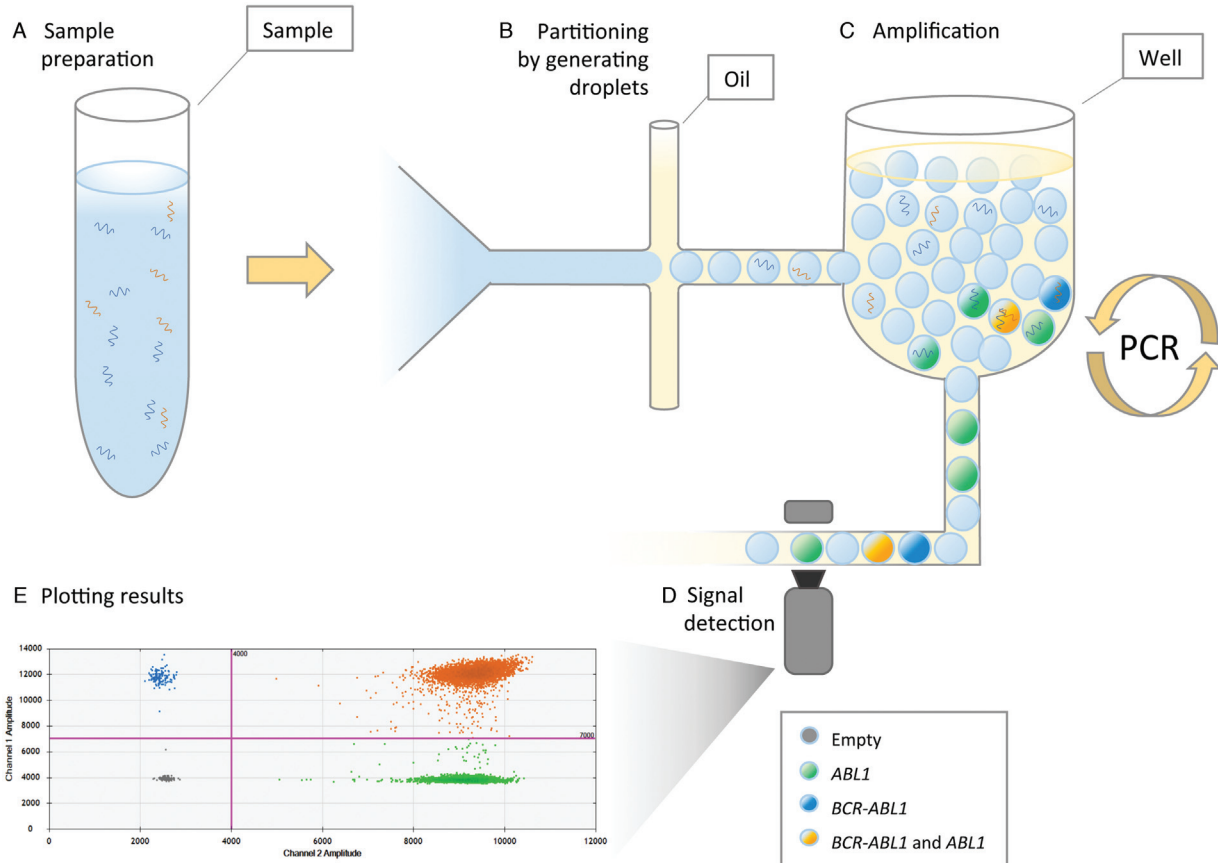


Figure 1. An overview of digital PCR with an example of a droplet-based platform with a duplex BCR-ABL1/ABL1 assay. (A) Sample preparation: RNA extraction, reverse transcription and addition of primer, probes, reaction mix (including surfactants); (B) sample partitioning into thousands of droplets using a droplet generator; (C) PCR amplification; (D) fluorescence signal detection per droplet in the droplet reader chip; (E) digital analysis and plotting results.

the detection of a single sequence in a single partition and is dependent on the available sample volume and the number of partitions.²² While in conventional analog qPCR assays uncertainty of detection is influenced by the resolution of the fluorimeter, in dPCR the uncertainty is mostly influenced by the occurrence of sampling and partitioning errors. Sampling errors are unavoidable and can occur in every diagnostic assessment of low-abundance target sequences, especially in clinical samples that are frequently more limited in size, that is, blood volume. Partitioning errors occur due to its statistical nature since distribution of targets among partitions can differ from one analysis to the next and diminish when using a higher number of partitions. On that part, much progress has been made in the available dPCR platforms, evolving from chamber partitioning (in a chip or plate) to microfluidic droplet-based platforms delivering an increasingly high number of partitions (droplet digital PCR or ddPCR; Fig. 1).²⁰ Alikian et al recently compared the various dPCR platforms in detail.²⁵ Furthermore, one sample can be analyzed in multiple wells (replicates), only limited by the amount of available sample. Current dPCR assays can achieve a LoD of 0.001% to 0.0001% in the detection of rare sequences.²⁰

Specificity depends on the occurrence of false positive events. False positives in dPCR are largely avoidable if using a well-optimized assay, since they arise from poor assay design, spurious amplification at higher PCR-cycle numbers and cross-contamination during the set-up. The hypothesis of sporadic presence of *BCR-ABL1* transcripts in healthy individuals is open for debate, but may contribute to the false positive rate (FPR) in negative controls.^{26,27} Reported FPR in dPCR assays is usually low to (preferably) non-existent (<5%).

Precision of the assay

Precision is one of the main assets of dPCR.²⁸ In comparative experiments, dPCR showed a greater inter-assay reproducibility measuring consistently <1.2-fold differences, while qPCR can only measure > 1.25-fold differences under ideal conditions.^{29–31} However, it must be taken into account that its intrinsic precision is influenced by partitioning errors just like the sensitivity and diminishes at the extremes of the assay's dynamic range.^{20,22} Inter-assay differences in distribution of target sequences especially occur when almost all partitions are empty or full (consistent with a very low or high average occupancy per partition). In the context of MRD measurements where precision is desirable even at low disease levels, it could be tempting to pre-amplify the sample to reach the optimal occupancy (the so-called "sweet spot" between an average concentration of 0.6 to 1.6 sequences per partition). However, the variation in pre-amplification efficiency is not consistent resulting in a semi-quantitative result and introducing a measurement bias that cannot be corrected accurately.³² Therefore, if a precise measurement is needed, direct quantification of low-level *BCR-ABL1* is still preferable using a high number of partitions and replicates to reduce partitioning errors and thereby maximizing precision.

Inter-assay differences can also occur due to additional factors such as molecular dropouts caused by damage to the target sequences (due to pre-analytical processes), variation in reverse transcription efficiency when RNA is used, or substantial PCR inhibition.³³ These pre-analytical variables need to be considered in both qPCR and dPCR analyses, affecting precision and sensitivity. Therefore, it is recommended to assess positive controls in parallel to the analyzed samples and to use a reference

sequence (eg, *ABL1* or *GUSB*) for quality verification and calibration.

Duplex digital PCR

The implementation of duplex (or multiplex) dPCR improved precision even more in the detection of two (or more) target sequences.^{34,35} In a duplex dPCR assay two target sequences are quantified using 2 distinct fluorescent probes in one reaction. In the context of CML, the second target sequence (eg, *ABL1* or *GUSB*) offers a quality control and the possibility to express the results as a ratio with *BCR-ABL1*. A more precise measurement is obtained by eliminating the differences in pre-analytical workup of 2 separate reactions and thereby corrects, for example, pipetting errors. Furthermore, it reduces the number of reactions, thus the costs and the amount of sample needed.

DNA-based approaches

Currently, reverse-transcribed *BCR-ABL1* RNA is mostly used for PCR quantification in CML. However, quantifying genomic DNA for MRD detection can offer several advantages over the RNA-based approach, including the stability and easy extraction of DNA, the elimination of variability due to the reverse transcription step and the possibility to detect leukemic cells regardless of transcriptional activity.³⁶ Especially the latter is drawing attention since it is known that some leukemic cells can enter a quiescent state and therefore evoke an underestimation of the disease burden when measuring the transcript.³⁷ However, the difficulty of mapping the genomic breakpoints of the *BCR-ABL1* translocation remains an undeniable obstacle. Breakpoints are distributed throughout several introns and often include additional deletions, insertions, duplications or inversions, requiring of a patient-specific assay design. Moreover, to design the assay, it is necessary to have access to a patient's sample with sufficient *BCR-ABL1* DNA material, that is, high leukemic load. This normally means that a sample taken at the time of diagnosis is needed, which is not always available. Despite the efforts to map all possible breakpoints more efficiently using different techniques, including next generation sequencing (NGS), the creation of libraries of usable primer and probes, the DNA-based approach is not widely adopted.³⁸

Digital PCR analytical performance in the context of CML

Precise and absolute quantification of *BCR-ABL1*

The main considerations regarding the sensitivity, specificity and precision of dPCR in general were highlighted in the previous section. Here, we will review a selection of studies assessing the analytical performance of dPCR specifically in the detection of *BCR-ABL1* transcripts, whether or not compared to conventional qPCR. The key findings are summarized in Table 2.

Goh et al were the first to describe dPCR for *BCR-ABL1* MRD measurement using a chamber partitioning platform, achieving a remarkable sensitivity down to 10^{-7} , thereby detecting *BCR-ABL1* in 24 of 32 qPCR negative samples (75%).³⁹ qPCR for *BCR-ABL1* and *ABL1* was performed in duplicate, samples contained at least 10.000 *ABL1* transcripts (equivalent to at least MR4.0 sensitivity) and qPCR negativity was confirmed with nested qPCR. Reliability of the results was evaluated by testing samples of healthy individuals and no-template controls (NTC),

Table 2**A Review of a Selection of Studies Assessing the Analytical Performance of Different Digital PCR Platforms for BCR-ABL1 Quantification in the Context of Chronic Myeloid Leukemia (CML).**

	Goh et al	Jennings et al	Andersen et al	Huang et al	Alikian et al	Franke et al	Chung et al
dPCR							
dPCR system	12.765 Digital Array™ (Fluidigm)	QX100™ (Bio-Rad)	QX100™ (Bio-Rad)	QX200™ (Bio-Rad)	(a) QuantStudio™3D (b) QX200™(Bio-Rad) (c) RainDrop™ (RainDance)	QX200™ (Bio-Rad)	QX200™ (Bio-Rad)
dPCR assay	Lab-developed	Lab-developed	Lab-developed	Lab-developed	Lab-developed	Commercial	Commercial
Maximal partitions per well (n)	765	20.000	20.000	20.000	(a,b) 20.000 (c) 10.000.000	20.000	20.000
Modality	Chamber	Droplet	Droplet	Droplet	(a) Chamber (b,c) Droplet	Droplet	Droplet
Pre-amplification	Yes	No	No	No	No	No	No
Performance characteristics							
Sensitivity							
Clinical detection limit (MR class)	MR7	MR6	MR5-MR5.5	MR5-MR5.5	(a,b) NR (c) MR5	MR5	NR
Wells combined to achieve detection limit	1	6	8	NR	NR	4	1
Specificity							
False positivity rate	0%	0%	NR	NR	NR	2%	0%
Precision							
Inter-assay variation	NR	MR3 CV=9% MR4 CV=16% MR5 CV=23% MR6 CV=112%	NR	NR	NR	MR3 CV=10% MR4 CV=37% MR4.5 CV=88%	MR3.5 CV=9.3%
Compared to qPCR							
Correlation coefficient (R ²)	0.98	NR	0.94	NR	(a) 0.87 – (b) 0.92 – (c) 0.97	0.98	0.996

CV = variation coefficient; LOD = limit of detection; MR = molecular remission; N/A = not applicable; NR = not reported.

in which no amplification was observed. The findings underscore that qPCR “negativity” should not be regarded as indicative for a complete molecular remission or cure. However, the sensitivity of 10^{-7} was only obtained if a pre-amplification step was used similar to nested PCR resulting in a semi-quantitative result reliable on pre-amplification efficiency and thereby less reproducible. Considering this, pre-amplification diminishes the advantages offered by dPCR especially in MRD measurements where precise and absolute quantification is desirable; therefore, it may not be recommendable to use it for *BCR-ABL1* quantification. The correlation between *BCR-ABL1* quantification by dPCR and qPCR was evaluated and found to be good at different MR-levels, except for samples with qPCR-*BCR-ABL1*-levels of $\geq 10\%$ IS (because of saturation of the platform) or $\leq 0.01\%$ IS (because of the lower sensitivity of dPCR since these samples were not pre-amplified).

Other studies investigating the sensitivity of dPCR in CML reported a clinical LoD varying between MR5.0 and MR6.0 achieved by combining multiple wells, which is a 1- to 2-log improvement compared to conventional qPCR.^{40–43} Only one study, comparing three different dPCR platforms, failed to demonstrate a LoD lower than MR5.0 because of a high FPR in all tested platforms causing interference of background noise with quantification of low-level disease.⁴⁴ The cause of the high FPR is not quite understood, especially since the other study groups reported low to non-existent false positives both in NTC as in negative controls.^{39,40,42,45} Even with this interference though, the accuracy of quantification at the level of MR4.0-MR5.0 was found to be better with dPCR.⁴⁴ The study of Franke et al investigated assay performance for 2 different assays and

also reported some false positives when using the Europe Against Cancer (EAC) primer and probe system requiring the use of a positivity threshold of three droplets to reduce background noise but thereby diminishing sensitivity.⁴² Performance characteristics were much better with a low FPR when using a commercially available assay, abolishing the need of a threshold and improving sensitivity (see Table 2).

The studies of Jennings et al and Franke et al assessed the precision of the assay by measuring multiple replicates by independent analysts on separate days and calculating its coefficient of variation (CV) as a marker of inter-assay variability, describing a better *BCR-ABL1* level of precision than with qPCR. Nonetheless, like with qPCR, dPCR’s variability increases with a decreasing transcript level.^{40,42} In low-level disease with concentrations beyond the LoD of a single well, some replicates will be positive and others negative, resulting in a substantially higher CV, as reflected in Table 2. An improvement in precision was achieved by combining the results of multiple wells; however, the resulting CV values were not numerically reported by the authors.

The study of Huang et al assessed the variation in dPCR results by using different primer and probes sets; and by using different dPCR platforms.⁴³ The variation caused by these changes was low, that is, less than two-fold differences were observed compared with 16- to 32-fold differences when using qPCR. This implies that dPCR assay performance is more robust than the qPCR assay.

In the study of Kjaer et al comparing qPCR and dPCR, a significant difference was found between the *BCR-ABL1* e13a2 and e14a2 variants when quantified using the widely applied

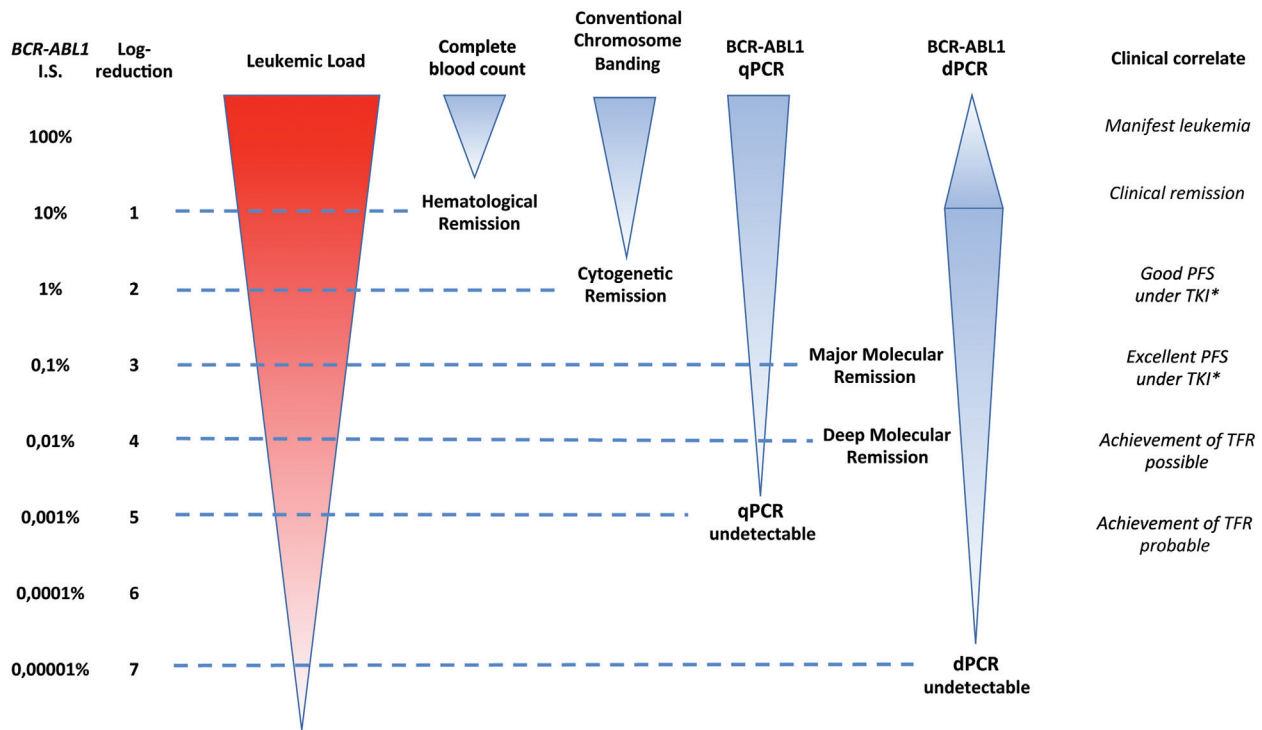


Figure 2. A representation of CML disease levels on the IS and their clinical correlate, with a comparison of the clinical detection limit of various diagnostic assessments to D-PCR. *PFS=progression-free survival. Patients achieving a complete cytogenetic response or major molecular response at 12 months had a good or excellent PFS at 24 months reported by Hughes et al in the IRIS-trial, that is, 95% and 100%, respectively.¹³

EAC qPCR assay, which was not seen in dPCR being an endpoint measurement.⁴⁶ This may be ascribed to a difference in PCR efficiency of the 2 variants in qPCR and could potentially artificially lower the IS qPCR values of the e14a2 variant by half a log. Using dPCR avoids this systematic error.

Comparison with real-time quantitative PCR

In the context of CML, we can conclude that dPCR offers at least a 1-log improvement in sensitivity, as well as an improved precision compared to conventional qPCR (see Fig. 2).^{39–43} Two different studies compared the clinical utility of dPCR to qPCR in monitoring MRD, by assessing consecutive samples of CML patients in durable deep molecular response (DMR) with both techniques. In the ENEST next study, dPCR detected *BCR-ABL1* in 40% of the MR4.5 qPCR negative samples and showed decreasing levels of *BCR-ABL1* with continued 2GTKI treatment.⁴⁷ In another study, dPCR was used to monitor DMR prior to a TKI discontinuation attempt, demonstrating a better quantification accuracy than qPCR, thus allowing a better selection of patients eligible for TKI discontinuation.⁴⁸

Besides the evolution of *BCR-ABL1* dPCR assays, there has also been major progress in optimizing qPCR assays. An automated cartridge-based *BCR-ABL1* assay has been introduced by Cepheid (Xpert[®] *BCR-ABL* Ultra) achieving a clinical detection limit reaching down to MR4.5 and diminishing qPCR's inter-assay variability.^{49,50} Precision of the Cepheid Xpert[®] *BCR-ABL* Ultra assay even approaches that of the QX200[™] dPCR commercial assay (Bio-Rad). Weighing the performance data reported by the manufacturers, the standard deviations (SD) were 0.16 and 0.06 log (average over all MR-classes); 0,33 (at MR4.3) and 0.25 log (at MR4.6) for the Xpert[®] *BCR-ABL* Ultra

assay and the QX200[™] dPCR commercial assay, respectively.^{51,52} Comparative studies in a clinical setting are needed. Especially comparative analysis of accuracy of qPCR vs dPCR assessing specifically the *BCR-ABL1* values set at the ELN milestones, would be of great added value since these are clinically relevant in treatment decision-making.

Precise MRD measurements of atypical *BCR-ABL1* transcripts

Another application for dPCR is molecular response monitoring in CML patients with atypical *BCR-ABL1* transcripts, first demonstrated by Zagaria et al and recently used by the study group of Petiti et al.^{53,54} After designing primers and probes flanking the different *BCR-ABL1* breakpoints of atypical transcripts, they used a multiplex dPCR assay in which all *BCR-ABL1* probes were labeled with one fluorochrome and the control gene *ABL1* probe with another. Their assay reliably quantified transcripts at all MR levels down to MR5.0.

Until now, response monitoring for these patients is performed almost exclusively by non-quantitative methods, such as qualitative nested PCR. They remain useful for diagnosis and identification of the atypical transcripts, however, their measurements are inaccurate at transcript levels below 1% failing to correctly identify MMR and excluding these patients from TKI discontinuation protocols. Petiti et al clearly demonstrate that dPCR outshines nested PCR especially for precise MRD measurements providing accurate molecular response information to help in delineating therapeutic options and perhaps select candidates for a TKI discontinuation attempt in this patient population.⁵³

Table 3**A Review of a Selection of Studies Assessing the Predictive Value of Digital PCR for Sustained TFR.**

	Mori et al (2015)	Lee et al (2016)	Bernardi et al (2018)	Colafigli et al (2019)	Nicolini et al (2019)
Study population					
Patients (n)	112	90	111	50	218
Main inclusion criteria	First line imatinib $\geq 2y$ RQ-PCR UMRD $\geq 1,5y$	First line imatinib $\geq 3y$ RQ-PCR UMRD $\geq 2y$	First line TKI RQ-PCR MR4, MR5 or UMRD $\geq 2y$	Outside clinical trial RQ-PCR MR4.5 or UMRD	First line imatinib $\geq 3y$ RQ-PCR UMRD $\geq 2y$
qPCR assay					
Clinical detection limit (MR class)	MR4 or better	MR5	MR5	MR4.5	MR4.7 or better
dPCR <i>BCR-ABL1</i> detection when qPCR undetectable	25/107 (23%)	16/88 (18%)	NR	22/50 (44%)	75/175 (43%)
dPCR assay					
dPCR system	12.765 Digital Array™ (Fluidigm)	12.765 Digital Array™ (Fluidigm)	QuantStudio™ 3D	QX200™ (Bio-Rad)	QX200™ (Bio-Rad)
Modality	Chamber	Chamber	Chamber	Droplet	Droplet
Maximal partitions per analysis (n)	765	765	40.000	60.000	40.000
Pre-amplification	Yes	Yes	No	No	No
Clinical detection limit (MR class)	MR7	MR7	MR5	NR	MR4.9
Positivity threshold	≥ 1 positive chamber ^a	≥ 17 positive chambers	≥ 1 positive chamber ^a	≥ 1 positive droplet ^a	$\geq 0,0013\%IS$
Prediction of TFR					
Optimal prediction cutoff	≥ 1 positive chamber ^a	≥ 17 positive chambers	$\geq 0,468$ copies/ μ l reaction volume	≥ 1 positive droplet ^a	$\geq 0,0023\%IS$
dPCR <i>BCR-ABL1</i> above cutoff	25/107 (23%)	16/88 (18%)	25/111 (23%)	22/50 (44%)	37/174 (21%)
Overall TFR rate	48%	59%	77%	65%	47%
TFR rate when dPCR <i>BCR-ABL1</i> above cutoff	32%	38%	52%	50%	32%
TFR rate when dPCR <i>BCR-ABL1</i> undetectable or below cutoff	57%	64%	86%	86%	54%
Difference in TFR rate statistically significant	Yes ($p=0,002$)	Yes ($p=0,021$)	Yes ($p=0,0003$)	Yes ($p=0,026$)	Yes ($p=0,0053$)
Other independent TFR predictors	Age ($>$ or $<$ 45y)	TKI withdrawal syndrome Treatment duration	DMR duration $>$ 5y	None found	Treatment duration

pts = patients; RQ-PCR = real-time quantitative PCR; TKI = tyrosine kinase inhibitor; UMRD = undetectable minimal residual disease.

^aassumed because not specified.

Digital PCR for prediction of sustained treatment-free remission

Achieving a TFR has become an important goal in the management of CML patients in stable DMR with a success rate of approximately 50% after a first TKI discontinuation attempt. New prognostic factors are needed to better predict the success thereby selecting the right candidates and improving this a priori chance. Up to date, only a longer duration of treatment and DMR were found and confirmed to be predictive. It would be intuitive to assume the depth of MRD is also a predictor for successful TFR; however, in the large EUROSKI trial no predictive value of MR class, that is, MR4.0 vs MR5.0 or undetectable disease, could be demonstrated when using qPCR.⁵ A possible explanation could be the poor precision and limited sensitivity of the qPCR assay resulting in a high variability in obtained low-level *BCR-ABL1* values. However, when using a dPCR *BCR-ABL1* assay, the MR-class did have a predictive value. Here, we present a review of five studies evaluating and all confirming the predictive role of dPCR (see Table 3). We will use the negative predictive value (NPV) of dPCR as comparative parameter, that is, the rate of sustained TFR in a dPCR negative (or below the cut-off) patient population.

Mori et al was the first study group assessing the predictive value of MRD in their ISAV trial, using a dPCR assay with chamber partitioning preceded by a pre-amplification step; previously described by Goh et al^{39,55} Enrolled CML patients

(n = 112) were treated with first-line imatinib and had undetectable MRD (UMRD) by qPCR (MR4.0 or better) for at least 18 months prior to TKI discontinuation. Despite qPCR negativity, almost 25% of patients showed a positive dPCR result. The relapse rate was higher in the dPCR positive group than in the dPCR negative group, demonstrating a NPV of 57% ($p=0,002$).

Lee et al had a similar approach in the KID trial using the same dPCR assay and almost identical inclusion criteria.⁵⁶ UMRD was defined as negative PCR results in a duplicate qPCR assay with 5-log sensitivity and to confirm UMRD the duplicate q-PCR assay was performed at six different time points. Among 88 patients with confirmed qPCR-UMRD, 16 (18%) had a positive result with dPCR at a cut-off of more than 17 positive chambers. They identified this cut-off performing a receiver operating characteristic curve (ROC) analysis for most optimal prediction of sustained TFR. Patients with dPCR-*BCR-ABL1* below the cut-off had a higher probability of sustained TFR than patients with dPCR-*BCR-ABL1* above the cut-off (64% vs 38%, $p=0,021$).

Bernardi et al assessed both the role of dPCR in MRD monitoring (as described previously) and its predictive value for sustained TFR.⁴⁸ All included patients had a durable DMR of MR4.0 or better for at least 2 years and were treated with TKIs for a median of 99 months. They followed a total of 111 patients attempting a TKI discontinuation both in clinical trials as in real-world practice dividing them with dPCR into 2 DMR classes at a cut-off of 0.468 *BCR-ABL1* copies/ μ l of reaction after ROC analysis. Patients with a DMR above this cut-off had a higher

relapse rate than patients below the cut-off: the NPV of DMR < 0.468 copies/ μ l was 86% for sustained TFR compared to 52% sustained TFR when > 0.468 copies/ μ l ($p=0.0003$).

Colafigli et al evaluated the predictive role of *BCR-ABL1* dPCR in 50 CML patients in durable undetectable MR4.5 stopping TKI treatment for the first time outside of a clinical trial.⁵⁷ In almost half of the qPCR negative samples, *BCR-ABL1* was detected by dPCR at very low concentrations with a median of 0.04 copies/ μ l. The rate of sustained TFR after TKI discontinuation was higher in the group with undetectable *BCR-ABL1* by dPCR (86%) compared to the group with detected *BCR-ABL1* (50%). Despite the relatively small patient population, this difference was significant ($p=0,026$).

The French study group of Nicolini et al conducted a stopping trial evaluating the cessation of first-line imatinib after at least 2 years of MR4.5 and assessing novel predictors including DMR determined by dPCR.⁵⁸ A total of 214 patients were enrolled, with a median follow-up after imatinib cessation of 23.5 months and an overall TFR success rate of 47%. Again the predictive value of a low or undetectable dPCR signal was demonstrated with a NPV of 54%, this time at a cut-off of 0.0023%IS determined with ROC analysis. Importantly, they calculated an assay-specific conversion factor through comparison with qPCR IS reference material, making it possible to define the cut-off value on the IS and thereby making the interpretation of the results of the dPCR assay suitable for extrapolation to other labs. Their reported cut-off value however has not yet been validated in other studies.

These five studies have a substantial heterogeneity in study design and applied dPCR assays. And although the negative and positive predictive values of dPCR varied between studies and were not perfect, the predictive value for sustained TFR was statistically and clinically significant in all of them.

Discussion: Is digital PCR applicable in clinical practice?

Molecular monitoring in CML is challenged by an increasing demand for better accuracy and sensitivity in *BCR-ABL1* quantification with a clinical emphasis on absolute quantitative targets set at timed milestones in response to TKI treatment (see Fig. 2). The currently used qPCR method is already at the limits of its technical possibilities to deliver these results. Therefore, dPCR may be introduced in today's clinical practice as a valuable substitute, since its improvement in both sensitivity and precision in *BCR-ABL1* quantification has clearly been demonstrated. Higher precision is crucial to reduce misclassifications in treatment responses. However, to make dPCR clinically applicable, a new effort of assay standardization is required and it is essential that dPCR *BCR-ABL1* results can be provided on the IS by using a laboratory-specific CF or by IS-standardization using the WHO IS reference material.^{43,58}

It remains to be established if dPCR can be developed further to increase its performance. The use of a pre-amplification step prior to dPCR can increase qualitative LoD, but quantitative results are not fully reliable as they cannot be corrected for the variation in pre-amplification efficiency.^{55,56} An alternative and preferable approach would be to increase assay performance by loading larger amounts of RNA sample and using a higher number of partitions.

Of course, meanwhile progress is seen in the qPCR assay as well. Comparative studies between optimized qPCR and dPCR

assays are still needed, for example, for their performance for *BCR-ABL1* values around the ELN milestones. Considering the costs, dPCR *BCR-ABL1* assay prices vary depending on the used platform and on the number of wells used per analysis.²⁵ Currently, using Bio-Rad's commercially available dPCR *BCR-ABL1* assay is more expensive than a regular in-house RT-qPCR assay, but prices fall in the same range as those for the automated cartridge-based Cepheid Xpert[®] assay. However, both the in-house RT-qPCR and dPCR require more hands on time compared to Cepheid Xpert[®] assay.

The increase in sensitivity makes dPCR also applicable for MRD monitoring and for the selection of patients with optimal chances of successful TKI treatment discontinuation, for both canonical and atypical *BCR-ABL1* transcripts.^{47,48,53} Only limited by the available sample size and the number of partitions, its sensitivity reaches levels of MR5.0 to MR6.0 with current protocols, which is a 1-log improvement compared to qPCR. While the depth of DMR assessed by qPCR was not predictive, several studies have shown a consistent predictive value of dPCR for successful TFR.^{5,48,55-58} To implement dPCR as a prediction tool in clinical practice, it is essential to define a reproducible cut-off value that can be used across different labs. The cut-off value defined by Nicolini et al meets these criteria and was set at 0.0023%IS, but has not yet been confirmed in other CML patient populations.⁵⁸

To conclude, dPCR can alleviate some inevitable shortcomings of qPCR. dPCR has clear benefits to reliably assess the achievement of treatment milestones after initiation of TKI therapy. In particular, dPCR is now established as a valuable tool to aid the selection and timing of TKI discontinuation.

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