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DROPLET DIGITAL PCR IS A ROBUST TOOL FOR MONITORING MINIMAL RESIDUAL DISEASE IN ADULT PHILADELPHIA-POSITIVE ACUTE LYMPHOBLASTIC LEUKEMIA

Coccaro N, Anelli L, Zagaria A, Casieri P, Tota G, Orsini P, Impera L, Minervini A, Minervini CF, Cumbo C, Parciante E, Carluccio P, Brunetti C, Specchia G, Albano F.

Affiliation:

Hematology Section, Department of Emergency and Organ Transplantation, University of Bari, Bari, Italy

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Corresponding author:

Francesco Albano, M.D., Hematology Section, Department of Emergency and Organ Transplantation, University of Bari, P.zza G. Cesare, 11, 70124 Bari, Italy.

e-mail: francesco.albano@uniba.it.

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Abstract

The *BCR-ABL1* p190 fusion transcript (m-bcr) is the most frequent variant observed in Philadelphia-positive (Ph+) acute lymphoblastic leukemia (ALL). Qualitative-PCR (QL-PCR) and real-time quantitative PCR (RQ-PCR) are the currently used methods to monitor minimal residual disease (MRD) in Ph+ ALL patients; for the latter, full standardization and an international quality validation are lacking. In this study, we developed a droplet digital PCR (ddPCR) assay for MRD monitoring in p190+ ALL cases. We assessed the analytical performance by the limit of detection (LOD) determination, demonstrating a reliability, sensitivity, and precision of the assay of up to 0.001%. Comparison of results obtained with QL-PCR and ddPCR in 117 follow-up samples from 16 of 26 Ph+ ALL patients showed discordant results in 27% cases (32/117). RQ-PCR analysis of 19 ddPCR positive samples with a low tumor burden failed to provide quantitative results in 63% cases (12/19). Our results highlight that in p190+ ALL the ddPCR method has a sufficient analytical performance for very low MRD monitoring and for predicting molecular relapse several months before hematological relapse. In conclusion, MRD monitoring by ddPCR may better stratify Ph+ ALL patients at risk of disease progression.

Introduction

Philadelphia-positive (Ph+) acute lymphoblastic leukemia (ALL) accounts for 20-30% of adult ALL (1-3). Cytogenetic and molecular qualitative analyses by PCR are usually employed to confirm the morphological diagnosis of Ph+ ALL and to assess which transcript variant is generated in each Ph+ ALL case (4). The *BCR-ABL1* p190 fusion transcript (m-bcr) is the most frequent variant being observed in 24% of ALL cases (3).

BCR-ABL1 tyrosine kinase inhibitors (TKIs) are considered an important component of treatment for adult patients affected by Ph+ ALL (5-7). In fact, recent studies reported an improved the overall outcome after treating Ph+ ALL patients with the combination of imatinib and multi-agent chemotherapy (8). Before the introduction of TKI therapy, Ph+ ALL patients had a poor prognosis, and a better outcome occurred mainly only in patients who underwent allogeneic stem cell transplant (allo-SCT) in first complete remission (CR) (9).

The investigation of residual *BCR-ABL1* transcript levels in Ph+ ALL patients shortly after starting TKI or during treatment is a strong prognostic factor, useful to detect early relapse and indicate a rapid change of therapy before hematological relapse (10-15). To date, minimal residual disease (MRD) monitoring of the p190 fusion transcript in Ph+ ALL patients has been performed by QL-PCR or by real-time quantitative PCR (RQ-PCR); this latter is a sensitive, easy to perform method but lacks full standardization and international quality validation (16, 17).

In this study we performed quantitative analysis of the *BCR-ABL1* p190 fusion transcript in 26 Ph+ ALL patients at diagnosis and during MRD monitoring at several follow-up time points by droplet digital PCR (ddPCR), a new, highly reproducible and precise PCR technology (18, 19) whose main advantage is that it enables accurate absolute quantification of target acid nucleic molecules without external references or standard curves. We compared the ddPCR performance with QL-PCR and RQ-PCR, proving that the ddPCR test is a highly sensitive method for monitoring *BCR-ABL1* transcript levels in Ph+ ALL cases, showing a remarkable reliability, sensitivity, and precision.

Methods

Patients

The study included 26 patients (median age 50 years, min. 24 – max 74 years; 10 males and 16 females) with newly diagnosed Ph+ ALL. The morphological diagnosis was confirmed in all cases by QL-PCR, karyotypic analysis and/or fluorescence in situ hybridization (FISH), as previously reported (20, 21) (Table 1). The median follow-up time of the entire cohort was 2.6 years (min. 4 months – max. 14 years). The main patients characteristics are reported in Table 1.

Molecular analysis

RNA extraction and reverse transcription

Total RNA was extracted from bone marrow samples at diagnosis or during the follow-up, using the QIAamp RNA Blood Kit (Cat#52304; Qiagen, Valencia, CA). The RNA concentration and purity were assessed using a NanoDrop spectrophotometer by NanoDrop Technologies (Wilmington, DE). For qualitative and quantitative real time experiments 1000ng of RNA samples were reverse transcribed into cDNA using the Standardized EAC RT protocol (23). For ddPCR experiments the iScript Advanced cDNA synthesis kit (Bio Rad) was employed; this kit offers a wide dynamic range for reverse transcription (from 100 fg to 7.5µg of RNA). Input RNA amount was optimized on the estimated *BCR-ABL1* abundance and sample availability; 1000ng and 7500ng of RNA were reverse transcribed for the samples at the onset and follow-up, respectively. Clinical samples at diagnosis from three patients and normal RNA samples from four healthy volunteers were mixed to create a single sample called *p190-pool* and *diluent-pool*, respectively.

Qualitative-PCR (QL-PCR) analysis

Identification of the *BCR-ABL1* fusion gene and of the e1a2 fusion transcript, corresponding to the p190 chimeric protein, was performed at diagnosis on bone marrow samples from all 26 ALL

patients according to the BIOMED-1 protocol (4). Samples at diagnosis were analyzed by first round PCR experiments with a sensitivity of about 10^{-3}, whereas a second round of amplification (nested PCR) with internal primers was performed on follow-up samples (sensitivity $10^{-3}/10^{-4}$) (4). The good quality of the cDNA sample was checked by verifying amplification of the housekeeping gene b-actin (*ACTB*). Qualitative results were classified as 'first positive', 'nested positive' or 'nested negative'.

Quantitative-PCR (RQ-PCR) analysis

RQ-PCR was performed on a LightCycler II 480 system (Roche Diagnostics, Monza, Italy), with the Ipsogen *BCR-ABL1* m-bcr Kit IVD kit (Qiagen). Absolute quantification was based on a standard curve built on five plasmid 10-fold dilutions (10^1, 10^2, 10^3, 10^5, and 10^5 copies) for the *BCR-ABL1* m-bcr transcript, and on three plasmid standard dilutions (10^3, 10^4, and 10^5 copies) for the *ABL* proto-oncogene 1 (*ABL1*) control gene. RQ-PCR analysis was conducted starting from 100ng of cDNA in triplicates for the fusion gene and in duplicates for the control gene. Results were interpreted according to the manufacturer's instructions for samples positive in all replicates and with an *BCR-ABL1*/*ABL1* ratio > 0.1; in particular, samples with an *ABL1* copy number > 1318 were considered acceptable for quantitative analysis. As well as CML, for samples with an undetectable *BCR-ABL1* transcript a minimum number of 10000 copies of the *ABL1* control gene for each replicate was required in order to classify the result as 'negative' (24, 25). Samples with a Cq higher than that of the lowest plasmid standard or with only one positive replicate were considered 'positive non quantifiable' (PNQ) (24, 25).

Droplet Digital PCR (ddPCR) analysis

Primers and Probes

In our study, ddPCR experiments were performed using primers and probes for *BCR-ABL1* p190 as previously described and employed in RQ-PCR (23). *GUSB* was used as control gene to confirm the

good quality of cDNA samples. Both *BCR-ABL1* p190 and *GUSB* primer and probes were used at a final concentration of 900 nmol/L and 250 nmol/L and were labeled with the FAM and HEX reporters, respectively. Each experiment included a negative (healthy donor) and a positive control sample (previously tested diagnostic specimen), as well as a no-template control. Results were analyzed when the number of accepted droplets per well was at least 10000 and the housekeeping gene provided correct (≥24000 *GUSB* transcripts (25, 26)) and reproducible amplification.

Determination of Limit of Detection (LOD)

To define the LOD for the *BCR-ABL1* p190 assay, the *p190-pool* was serially diluted into the *diluent-pool*. A 10-fold serial dilution was performed with the following concentrations: 10^{0}, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}. The *diluent-pool* was also used as negative control. A total of six replicates using 750ng of cDNA template were performed for each point of the serial dilution except for the lowest concentration (10^{-5}, 0.001%), which was tested 9 times, and the negative sample, which was tested in 21 replicates (27).

ddPCR experiments and data analysis

BCR-ABL1 expression analysis by ddPCR was performed in 26 Ph+ ALL patients at disease onset and in 16 of 26 patients during the follow-up. The *BCR-ABL1* and *GUSB* transcripts were analyzed in multiplex in the same well for the samples at diagnosis; by contrast, for the follow-up samples, the two genes were tested separately in different wells because different quantities of cDNA were needed. In follow-up samples, in fact, the analysis was performed on a larger quantity of cDNA, to allow the detection of rare copies of the *BCR-ABL1* fusion transcript. ddPCR experiments were performed using the QX-200 instrument (BioRad); this system combines water-oil emulsion droplet technology with microfluidics. Each sample is partitioned into about 20000 droplets by a droplet generator and each droplet is amplified by PCR. Then, droplets are streamed in single file on a droplet reader, which counts the fluorescent positive and negative droplets to define the target concentration. Fifty ng of cDNA template were used for the samples at the onset, and 750ng for follow-up samples in a final volume of 20 uL. In order to increase the sensitivity, three replicates

were run for follow-up samples; the *GUSB* transcript was run in a separate well using 100 ng of template. The 20-uL ddPCR reaction mixture was then loaded into the Bio-Rad DG8 droplet generator cartridge. A volume of 70 uL of droplet generation oil was loaded for each sample. The cartridge was placed in the QX200 droplet generator. The generated droplets were transferred to an Eppendorf 96-well PCR plate (Eppendorf, Hamburg, Germany). The plate was sealed with a BioRad pierceable foil heat seal, and samples were amplified on the T100 BioRad thermal cycler. Thermal-cycling conditions were 95 °C 10 minutes (1 cycle), 94 °C 30 seconds (ramp rate 2 °C/second, 40 cycles), 60 °C 1 minute (ramp rate 2 °C/second, 40 cycles), 98 °C 10 minutes (1 cycle), and 4°C hold. After amplification, the 96-well PCR plate was loaded on the Bio-Rad QX200 droplet reader and ddPCR data were analyzed with QuantaSoft analysis software (version 1.7.4). The target concentration in each sample was expressed as *BCR-ABL1* copies/μg.

Results

The analytical performance of the *BCR-ABL1* p190 ddPCR assay was defined by the LOD determination, using a pool of samples from three ALL patients at diagnosis, *p190-pool*, (cases #7, #20, and #22) and a *diluent pool* of samples from healthy controls. Data from serial dilutions showed remarkable linearity, reliability, and a precision of up to 0.001% (Figure 1A-B, Table 2). After converting to log-log scale, linear regression showed no concentration-dependent bias, and R^2 equaled 0.996 (Figure 1A-B). Because the negative samples showed no background, even a single detected droplet was defined as significant.

Overall, all the 26 Ph+ ALL at the onset and 253 follow-up samples were evaluable by QL-PCR: 34% were first positive (95/279), 27% nested positive (76/279), and 39% nested negative (108/279). ddPCR experiments were successfully performed in all 26 ALL cases at disease onset; during follow-up monitoring, a total of 117 follow-up time points were analyzed by ddPCR in 16 of 26 patients (Figure 2).

The analysis performed on follow-up samples showed concordant ddPCR and QL-PCR results in 98% of cases (46 ddPCR positive out of 47 first/nested positive samples); in detail, the only discordant result was obtained for a nested positive sample that resulted ddPCR negative (Figure 3). On the contrary, among the 70 nested negative follow-up points, 39 (56%) also resulted negative at ddPCR analysis (Figure 3). Follow-up points that were negative in ddPCR remained negative even when the experiments were repeated. For positive samples, quantitative results remained the same when the experiment was repeated and when the cDNA amount of three wells was loaded into a single well.

The amount of *BCR-ABL1* fusion transcript at the disease onset resulted very heterogeneous showing a median concentration of $101x10^3$ *BCR-ABL1* copies/µg (min $3.24x10^3$ – max $1744x10^3$ copies/µg). Of note, ALL patients that presented an adjunctive Ph chromosome or a *BCR-ABL1* gene amplification (#6, #8 and #9, Table 1) showed a high transcript amount; in fact, the median value of these latter cases was statistically different from the value calculated for the entire cohort (376.8 vs 106 *BCR-ABL1* copies/µg, respectively, p = 0.02) (Figure 4). For nested positive follow-up samples the median value was 30 copies/µg (min 0.5 – max 230 copies/µg).

Among the 70 sequential nested negative points, 31 (44%) resulted positive at ddPCR analysis (median value 2.62 copies/µg, min 0.27 – max 34.58 copies/µg) (Figure 2, green square and yellow circle). In order to further investigate p190 ddPCR assay reliability, and to see whether discordances were due to a minor sensitivity of QL-PCR, 19 ddPCR positive cases with a very low *BCR-ABL1* copy number (9 nested positive and 10 nested negative; median value 7 copies/µg) and 1 ddPCR negative but nested positive case were selected for RQ-PCR experiments: 7/20 (35%) cases were quantifiable, 3/20 (15%) were negative and 10/20 (50%) were classified as PNQ (Figure 5). Among the 10 nested positive cases, 5 were positive and quantifiable, 3 were PNQ and 2 were negative; the 10 nested negative follow-up points were quantifiable in 2 cases, 7 were PNQ, and 1 resulted negative (Figure 5). Of note, the 7 RQ-PCR quantifiable cases showed the highest values of *BCR-ABL1* copies (median value 15 copies/µg, min 7 - max 45 copies/µg) at ddPCR. Overall, for 16/20

cases (80%) concordant positive ddPCR and RQ-PCR results were obtained; in the remaining four cases, 3 (15%) ddPCR positive cases resulted RQ-PCR negative and the one (5%) ddPCR negative case was PNQ by RQ-PCR (Figure 5).

Comparing results obtained with the different molecular techniques, it is clear that in at least 13 follow-up points ddPCR positivity anticipated molecular relapse detected by QL-PCR (Figure 2, black arrows). Among the RQ-PCR data of the above-mentioned follow-up points, two cases were quantifiable (#5, follow-up point 1, 0.084% *BCR-ABL1/ABL1*; #7, follow-up point 4, 0.09% *BCR-ABL1/ABL1*), one case resulted negative (#4, follow-up point 9) and the remaining two points resulted PNQ (#10, follow-up point 5 and #13, follow-up point 7).

Discussion

Treatment of Ph+ ALL adult patients with TKIs has substantially improved the disease outcome, allowing the achievement of complete response (CR) rates in 95–100% of treated patients, although most of them relapse in a short time, even after hematopoietic SCT (11, 12, 28-32). In most laboratories, MRD molecular monitoring in Ph+ ALL patients is carried out by RQ-PCR; however, quantification of the *BCR-ABL1* p190 fusion transcript still lacks a standardized procedure to perform experimental analysis and to express final results. The standardization of experimental and analytical procedures achieved good results in chronic myeloid leukemia (CML) for *BCR-ABL1* p210 transcript monitoring, both adopting the use of calibrators and laboratory conversion factors (26, 33-35). However, the performance of *BCR-ABL1* transcript quantification may vary considerably across different laboratories because of a large variability inherent in the RQ-PCR technology (36).

Nowadays, ddPCR is the most sensitive method for determining the absolute quantification of nucleic acids both at disease diagnosis and during follow-up. As compared to RQ-PCR technology, digital PCR has several advantages as it identifies the absolute copy number of nucleic acid target

without the need for calibrators or standard curves. Digital PCR is especially powerful in MRD monitoring, as it is able to detect rare mutant events in a background of wild-type molecules.

In fact, several researchers have endeavored to investigate the reliability of ddPCR in MRD monitoring of hematological malignancies (37-44). To date, only one study employed digital PCR technology for MRD monitoring of Ph+ ALL patients, comparing its performance and sensitivity to the method based on RQ-PCR (37). In this study a microfluidic digital PCR approach was used (Biomark system from Fluidigm) and 60 *BCR-ABL1*+ ALL samples in hematologic and cytogenetic remission were analyzed. However, as compared to the digital PCR technologies available today, the Fluidigm system was less sensitive and powerful, as only about 9000 partitions could be generated. Moreover, no quantitative analysis was performed on Ph+ ALL patients at diagnosis. A recent study reported many advantages of monitoring the *BCR-ABL1* p210 fusion transcript by ddPCR with Bio-Rad's QX100 system, as compared to RQ-PCR in CML patients (27).

In our report we performed absolute quantification of the *BCR-ABL1* p190 fusion transcript by ddPCR with the Bio-Rad QX-200 system in 26 Ph+ ALL patients both at disease onset and during follow-up. The purpose was to investigate the analytical applicability of the ddPCR technique and to define its possible predictive molecular value during Ph+ ALL follow-up.

We developed a sensitive ddPCR assay showing a LOD of less than 0.001%, able to quantify small transcript levels by loading a high quantity of cDNA in different wells and combining the counts from multiple replicates. Of note, scaling up the ddPCR reaction to 750 ng of cDNA did not have a negative impact on the reaction performance.

Comparison of results performed by the QL-PCR and ddPCR on 117 follow-up samples from 16 of 26 Ph+ ALL patients showed concordant results in 85/117 (73%) cases. Concordance was 98% for first/nested positive samples (46 ddPCR positive of 47 first/nested positive follow-up points); instead, for nested negative cases it decreased to 56% (39 ddPCR negative of 70 nested negative samples). Taken together, discordant results were found in 32/117 (27%) cases: 1 case was positive

at QL-PCR but negative at ddPCR, and 31 samples were positive at ddPCR but negative at QL-PCR.

Comparison between RQ-PCR and ddPCR in 19 samples with a low tumor burden and 1 ddPCR negative case showed concordant positive results for 16 (80%) cases, even if 9 cases with very low amounts of *BCR-ABL1* were positive but not quantifiable (PNQ) by the RQ-PCR test. Furthermore, 3 ddPCR positive cases resulted totally negative at RQ-PCR and one case was PNQ at RQ-PCR but ddPCR negative; taken together, RQ-PCR failed to provide quantitative results in 12/19 (63%) of cases. These data highlight a gap between the sensitivity and the quantitative range for RQ-PCR p190 assay, which is a critical limitation, as borderline samples that fall in the range of inadequate quantification cannot be categorized. ddPCR appears to be a good alternative method to fill this gap. Of note, in one case (Figure 2, #3, follow-up point 14) QL-PCR and RQ-PCR were positive, but ddPCR was negative; this discrepancy could probably be due to an extremely low level of disease that could not guarantee reliability during the reverse-transcription phase.

All patients in our series experienced molecular relapse by qualitative PCR. In 13 follow-up samples, ddPCR could have identified tumor clone recurrence several months before qualitative analysis. This is particularly evident for patients #10, #11, #12 and #13, who had hardly any follow-up samples resulting molecular negative by ddPCR (Figure 2, black arrows). These results demonstrate that the ddPCR method has a sufficient sensitivity to predict molecular relapse detectable by QL-PCR and/or RQ-PCR from a few months to many years before hematological relapse, and could potentially offer a temporal advantage in the management of patients, improving the chance of modulating therapy and maybe preventing disease progression.

Our data prove that QL-PCR is an inadequate method for monitoring MRD in p190 Ph+ ALL cases, as it cannot reveal a very low transcript burden. On the contrary, RQ-PCR, the current quantitative method, offers the advantage of a higher sensitivity, but exploits relative quantification and does not provide adequate quantification for samples with a tumor burden at the limit of quantification. These data are consistent with results obtained in other hematological neoplasms (39, 42, 44-48).

In patients with Ph+ ALL, failure to achieve MRD negativity is associated with poor outcomes; in this context allo-SCT should be strongly considered (13). On the contrary, the achievement of MRD negativity may obviate the need for allo-SCT for many patients; in fact, it has been reported that in Ph+ ALL patients treated with chemotherapy plus a TKI, a deeper molecular response was achieved associated with an improved survival (14, 17, 49). Moreover, two recent reports described an excellent long term survival in Ph+ ALL patients who received hyper-CVAD plus a TKI, achieved a deep molecular response and did not undergo allo-SCT in first CR (12, 13). In view of the association between MRD response and outcomes in Ph+ ALL, it is reasonable to think that an improvement in MRD detection, as obtained with ddPCR technology, might help to better select patients for allo-SCT.

In conclusion, ddPCR is a rapid and sensitive method for performing absolute quantification of the *BCR-ABL1* p190 fusion transcript both at ALL diagnosis and during MRD assessment in routine clinical laboratories, without the need for calibrators, standard curve, and laboratory conversion factors. Its application in a larger cohort of patients will better define its clinical predictive value in MRD monitoring.

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Tables

Table 1. The main characteristics of the Ph+ ALL patients.

Case	Age/Sex	WBC ($x10^9$/L)	Cytogenetic analysis (ref.22)	FISH analysis (ref.22)	QL-PCR (ref.4)
#1	24/F	173	46,XX,t(9;22)(q34;q11)[20]	N/A	m-bcr (p190 BCR-ABL1)
#2	64/M	14,92	N/P	nuc ish(ABL1,BCR)x3(ABL con BCRx2)[400]	m-bcr (p190 BCR-ABL1)
#3	61/F	5,97	N/A	ish t(9;22)(ABL1+,BCR+;BCR+,ABL1+)[20]	m-bcr (p190 BCR-ABL1)
#4	62/M	303	N/P	nuc ish(ABL1,BCR)x3(ABL con BCRx2)[400]	m-bcr (p190 BCR-ABL1)
#5	33/F	66	N/A	nuc ish(ABL1,BCR)x3(ABL con BCRx2)[400]	m-bcr (p190 BCR-ABL1)
#6	50/F	78,6	46,XX,der(9)t(2;9)(p?;p?)t(9;12;22)(q34;p?13;q11)[4]/49,XX,+der(2)t(2;9)(p?;p?),+8,der(9)t(2;9)(p?;p?)t(9;12;22)(q34;p?13;q11),+22[16]	nuc ish(ABL1,BCR)x5(ABL con BCRx4)[400]	m-bcr (p190 BCR-ABL1)
#7	41/M	24,5	46,XY,del(9p),t(9;22)(q34;q11)[16]/46,XY[4]	N/A	m-bcr (p190 BCR-ABL1)
#8	69/F	3,26	45~46,XX,add(2)(q37),der(5q),-7,t(9;22)(q34;q11),12p-,-?14,?der(21),+?Ph[cp19]/46,XX[1]	ish t(9;22)(ABL+,BCR+;BCR+,ABL1+)der(22)(BCR+,ABL1+)[18/20]	m-bcr (p190 BCR-ABL1)
#9	51/F	26	47,XX,t(2;12)(q?31;q?22),t(9;22)(q34;q11),+der22t(9;22)(q34;q11)[8]/94,XXXX,t(2;12)(q?31;q?32)x2,t(9;22)(q34;q11)x2,+der(22)t(9;22)(q34;q11)x2[4]/46,XX[8]	ish t(9;22)(ABL+,BCR+;BCR+,ABL1+)der(22)(BCR+,ABL1+)[9/20]/ t(9;22)(ABL+,BCR+;BCR+,ABL1+)x2der(22)(BCR+,ABL1+)x2[3/20]	m-bcr (p190 BCR-ABL1)
#10	74/F	2,1	N/A	N/A	m-bcr (p190 BCR-ABL1)
#11	30/F	8,49	46,XX,t(9;22)(q34;q11)[14]/46,XX[6]	N/A	m-bcr (p190 BCR-ABL1)
#12	59/F	2	N/A	ish t(9;22)(ABL1+,BCR+;BCR+,ABL1+)[20]	m-bcr (p190 BCR-ABL1)
#13	43/F	20,7	N/A	ish t(9;22)(ABL1+,BCR+;BCR+,ABL1+)[20]	m-bcr (p190 BCR-ABL1)
#14	32/M	42,9	N/A	N/A	m-bcr (p190 BCR-ABL1)
#15	42/M	17,3	N/A	N/A	m-bcr (p190 BCR-ABL1)
#16	71/M	16,43	N/A	N/A	m-bcr (p190 BCR-ABL1)
#17	32/M	4,52	N/A	N/A	m-bcr (p190 BCR-ABL1)
#18	45/F	4,6	N/A	ish t(9;22)(ABL1+,BCR+;BCR+,ABL1+)[20]	m-bcr (p190 BCR-ABL1)
#19	42/M	29,3	N/A	N/A	m-bcr (p190 BCR-ABL1)
#20	27/F	27,45	N/A	N/A	m-bcr (p190 BCR-ABL1)
#21	68/F	9,79	N/A	N/A	m-bcr (p190 BCR-ABL1)
#22	69/F	8	N/A	N/A	m-bcr (p190 BCR-ABL1)
#23	29/M	2,6	N/P	nuc ish(ABL1,BCR)x3(ABL con BCRx2)[400]	m-bcr (p190 BCR-ABL1)

#24	65/F	23,1	44,XX,-8,t(9;22)(q34;q11),-15,?add(20q)[20]	N/A	m-bcr (p190 BCR-ABL1)
#25	58/M	113,6	44,XY,-8,t(9;22)(q34;q11),-11,-14,der(16)t(8;16)(q?11.2;q?12),add(17p),der(20)t(14;20)(q?11.2;q?11.2),+mar[10]/46,XY[10]	N/A	m-bcr (p190 BCR-ABL1)
#26	50/F	3,83	45,XX,-7,t(9;22)(q34;q11)[20]	N/A	m-bcr (p190 BCR-ABL1)

F: female ; M: male

N/P: not performed because of the sample quality

N/A: not avaible

Table 2. Analytical performance parameters of the ddPCR p190 assay: cDNAs from three pre-treatment samples were pooled (*p190-pool*) and diluted in total cDNA from healthy donors (*diluent-pool*). SD: standard deviation.

N.r of replicates	Dilution series (target value - %)	Data mean (copies/µg)	SD
4	10^{0} (100%)	144300	3897
6	10^{-1} (10%)	14056	482
6	10^{-2} (1%)	1315	33
6	10^{-3} (0.1%)	152	19
6	10^{-4} (0.01%)	17.4	3.5
9	10^{-5} (0.001%)	3.8	1.9
21	0	0	0

Figure legend

Figure 1. BCR-ABL1 p190 ddPCR assay Limit of Detection (LOD) determination by serial dilutions. A. The FAM fluorescent signal of each droplet is plotted against the droplet cumulative count. The magenta line indicates the positive fluorescence threshold. The blue dots represent individual droplets containing at least one copy of the transcript. Positive droplets were observed at dilutions as low as 0.001%. **B.** Log transformations of *BCR-ABL1* copies/µg and of target values show linearity and lack of bias. R^2 nearly equal to 1 indicates a remarkable correlation and no concentration-dependent bias.

Figure 2. Monitoring of minimal residual disease (MRD) levels in 16 of 26 Ph+ ALL patients performed by qualitative-PCR (QL-PCR – square), droplet digital PCR (ddPCR – circle) and real-time quantitative-PCR (RQ-PCR – triangle). Comparison of results obtained with QL-PCR and ddPCR in 117 follow–up samples showed discordant results in 27% cases (32/117): 31 QL-PCR negative but ddPCR positive cases, and 1 ddPCR negative but QL-PCR positive case (*). Black arrows indicate follow-up points with discordant QL-PCR/ddPCR results, for which ddPCR could have identified tumor clone recurrence several months before qualitative analysis.

#1, follow-up point 2 [interval between points 2-3=1 month];

#3, follow-up point 3 [interval between points 3-5=6 months];

#4, follow-up point 9 [interval between points 9-10=9 months];

#5, follow-up point 1 [interval between points 1-3=4 months];

#7, follow-up point 4 [interval between points 4-5=1 month] and follow-up point 11 [interval between points 11-12=3 months];

#8, follow-up point 4 [interval between points 4-7=7 months];

#9, follow-up point 2 [interval between points 2-3=2 months];

#10, follow-up point 5 [interval between points 5-7=9 months];

#11, follow-up point 7 [interval between points 7-8=3 months];

#12, follow-up point 7 [interval between points 7-11=13 months];

#13, follow-up point 7 [interval between points 7-9=3 months] and follow-up point 13, [interval between points 13-16=11 months]).

Cross indicates deaths. Numbers stated for sequential follow-up points do not indicate a specific temporal value.

Figure 3. Histograms showing comparison of minimal residual disease (MRD) analysis by QL-PCR (first/nested positive, nested negative) and ddPCR in 117 follow-up samples from 16 of 26 ALL patients. In 98% of QL-PCR positive cases concordant results were obtained with the ddPCR test (46 ddPCR positive of 47 first/nested positive samples); among the nested negative follow-up points, 39 of 70 (56%) also resulted negative at ddPCR.

Figure 4. Graph indicating *BCR-ABL1* absolute copy numbers (copies/µg). Ph+ ALL patients with an adjunctive Ph chromosome or a *BCR-ABL1* gene amplification (#6, #8 and #9) presented higher transcript amounts, with a statistically different median value from the value calculated for the entire cohort (106 vs 376.8 *BCR-ABL1* copies/µg, respectively, p = 0.02).

Figure 5. Comparison between RQ-PCR and ddPCR for 20 follow-up points (10 positive and 10 negative at QL-PCR). The analysis showed that for 16 (80%) cases RQ-PCR and ddPCR gave concordant positive results, even if 9 cases with very low amounts of *BCR-ABL1* were positive but not quantifiable (PNQ) by the RQ-PCR test. Three ddPCR positive cases were negative at RQ-PCR and one QL-PCR positive case resulted PNQ at RQ-PCR, but negative at ddPCR. In 12/19 (63%) ddPCR positive cases RQ-PCR failed to give a quantitative result.

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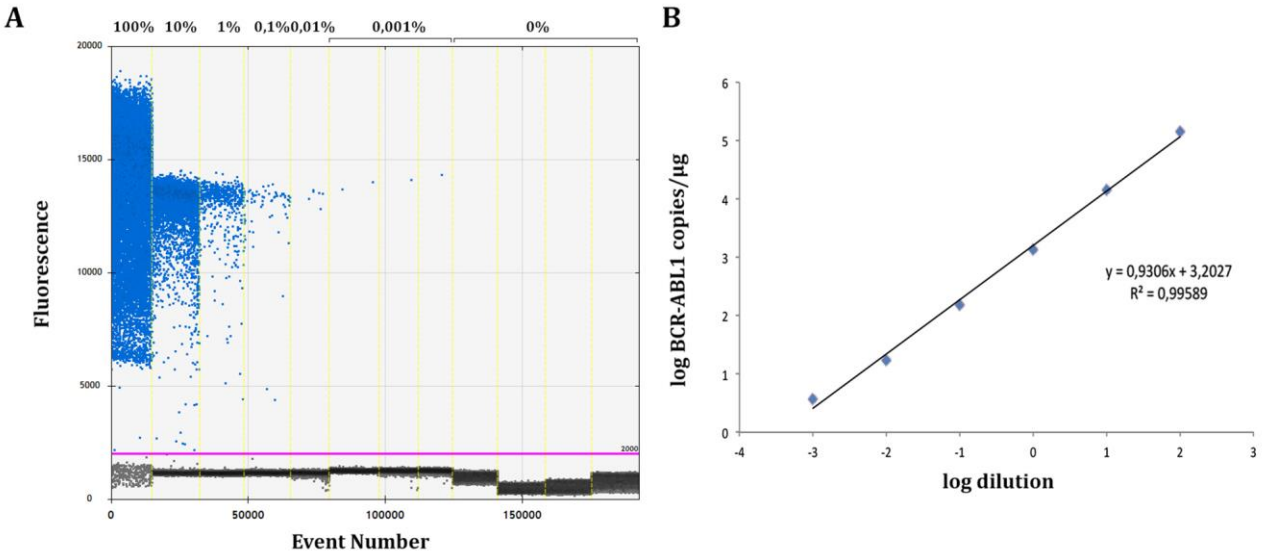

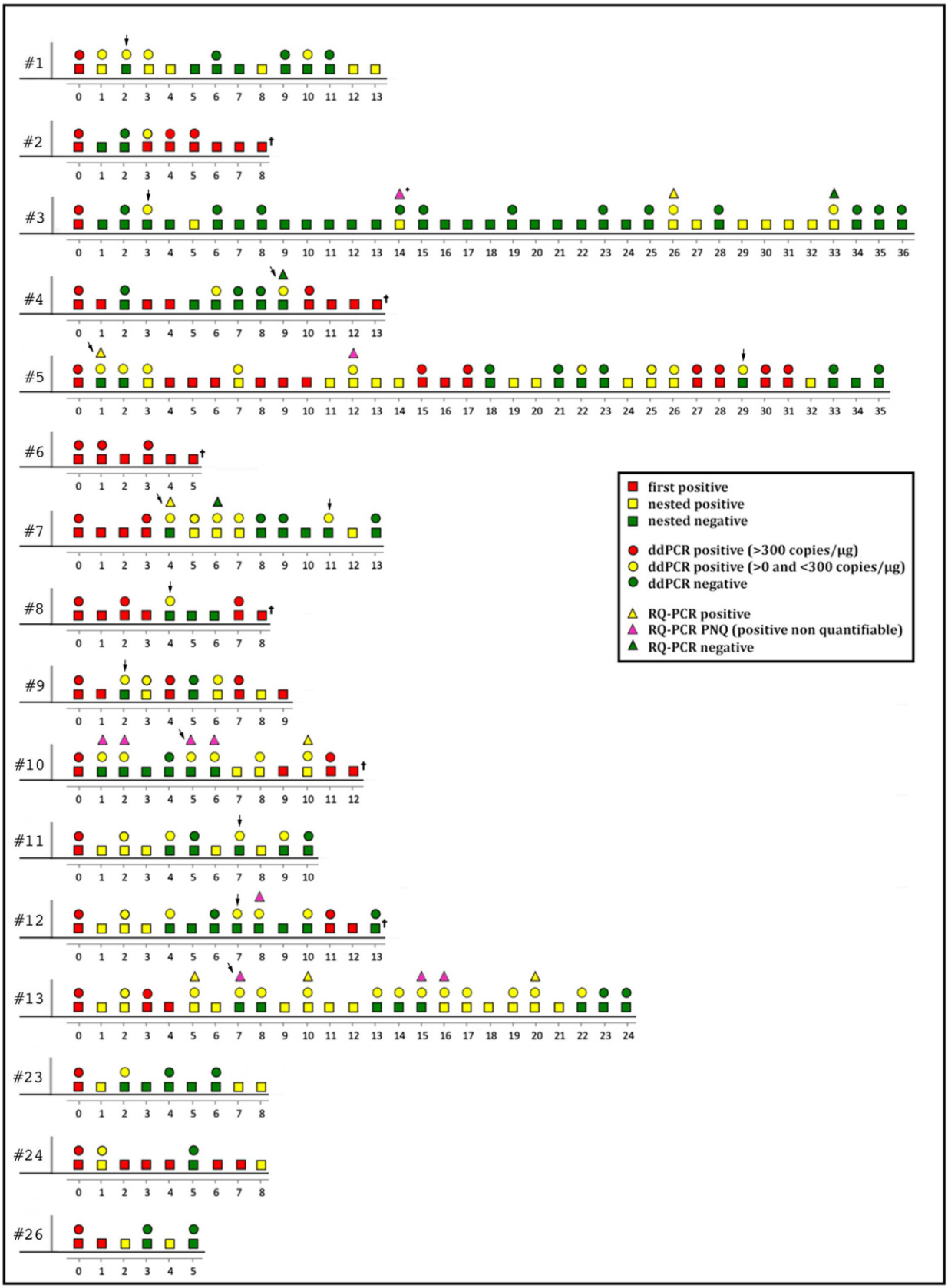
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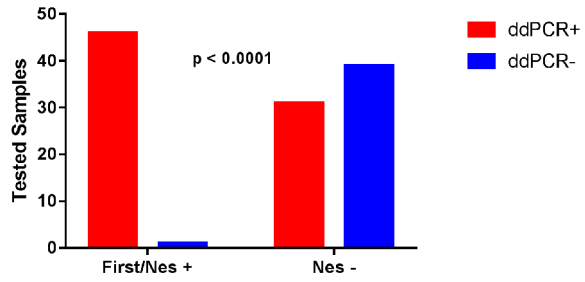
first positive
nested positive
nested negative
ddPCR positive (>300 copies/µg)
ddPCR positive (>0 and <300 copies/µg)
ddPCR negative
RQ-PCR positive
RQ-PCR PNQ (positive non quantifiable)
RQ-PCR negative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Tested Samples
p < 0.0001
ddPCR+
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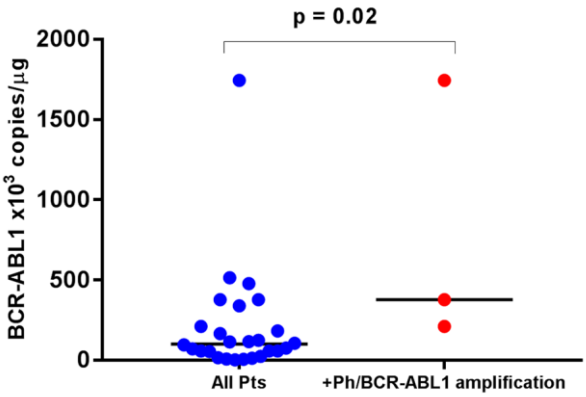
First/Nes +
Nes -


p = 0.02
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1500
1000
500
0
BCR-ABL1 x10³ copies/µg
All Pts
+Ph/BCR-ABL1 amplification


nested PCR
RQ-PCR
5 positive
2 negative
3 PNQ
RQ-PCR
2 positive
1 negative
7 PNQ
ddPCR positive
ddPCR negative