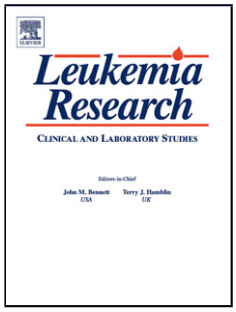

Title: Use of a High Sensitive Nanofluidic Array for the Detection of Rare Copies of *BCR-ABL1* Transcript In Patients with Philadelphia-Positive Acute Lymphoblastic Leukemia in complete response

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Leukemia Research

Use of a High Sensitive Nanofluidic Array for the Detection of Rare Copies of *BCR-ABL1* Transcript In Patients with Philadelphia-Positive Acute Lymphoblastic Leukemia in complete response

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Key words: *BCR-ABL1*, minimal residual disease, leukemia

Short running title: dPCR for monitoring MRD in leukemia

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Abstract

Monitoring of minimal residual disease (MRD) by quantification of *BCR-ABL1* transcript levels has become a main part of the management of patients with *BCR-ABL1*-positive acute lymphoblastic leukemia (ALL) in treatment with tyrosine kinase inhibitors (TKIs). The failure to achieve molecular negativity shortly after starting TKI has been demonstrated to be predictive of relapse, suggesting that an accurate measurement of low *BCR-ABL1* levels may have a role in preventing hematological relapse. Despite the big efforts made by many European laboratories within the European Study Group, at the time of writing a standardized procedure to quantify and express results is still missing for *BCR-ABL1*-positive ALL. In this study, in order to detect with high sensitivity low levels of *BCR-ABL1* transcripts, we used a new technology and a new molecular approach based on microfluidic digital polymerase chain reaction (dPCR) using Taqman chemistry and we compared obtained results with those generated by the conventional method based on reverse transcriptase PCR reaction (RQ-PCR) for *BCR-ABL1* and total *ABL1*, with TaqMan chemistry and with Applied Biosystems instrument. We demonstrated the dPCR is high-sensitive (able to detect a single copy of *BCR-ABL1*) and reliable (results are comparable to those obtained by *BCR-ABL1* quantification with conventional technology), allowing an accurate monitoring of *BCR-ABL1*-positive ALL patients in complete remission.

Introduction

The *BCR-ABL1* transcript resulting from the t(9;22) chromosome translocation known as the Philadelphia (Ph) chromosome is the most frequent genetic abnormality associated with adult ALL. Treatment strategies based on TKIs of first and second generation have substantially improved overall treatment results, with rapid and complete response (CR) rates in 95-100% of patients [1-6]. Nevertheless, the majority of them experience hematological relapse in a short time, also after hematopoietic stem cell transplantation (SCT) [7]. The presence of *BCR-ABL1* transcripts after alloSCT in the pre-imatinib era was indicative of minimal residual disease (MRD) and predicted a relapse in patients with *BCR-ABL1*-positive ALL [8]. Thereafter in the TKI era, the failure to achieve molecular negativity shortly after starting imatinib was predictive of relapse [9-10]. Moreover, *BCR-ABL1* levels lower than 10^{-3} at day 85 have been demonstrated to correlate with higher disease-free survival compared with patients who never reached these levels during induction of dasatinib treatment [6]. Therefore, the detection of residual *BCR-ABL1* transcript levels by quantitative polymerase chain reaction (qPCR) provides relevant clues to detect an early relapse during TKI treatment therapy allowing a prompt switch of therapy before hematological relapse. The monitoring of residual *BCR-ABL1* transcript levels has been recently well standardized for p210 quantification in chronic myeloid leukemia (CML) [11]. The establishment of a laboratory-specific conversion factor using a process initiated by the Adelaide laboratory allows to report own molecular results on an international scale, which standardizes quantitative *BCR-ABL1* measurements across tests and laboratories, allowing multiple laboratory studies, patient management, and a harmonized definition of treatment response [12]. In contrast to p210, there is less standardization for p190 quantification. The European Study Group's is currently performing twice annual quality control rounds to define a pan-European standard, but at the time of writing

there is still variation in methodology and reporting results among different participating laboratories. In recent years, new technologies have emerged to provide a very sensitive detection of very low levels of disease by microfluidic digital PCR (dPCR). In dPCR single molecules are isolated by dilution and individually amplified by PCR. The partitioning of the sample prior to PCR amplification in chambers containing 0 or 1 copy of target DNA allows that each product is analyzed separately. During analysis a Poisson correction is applied to the results to account for chambers that contain more than one molecule, and an absolute target sequence quantity is estimated [13]. Among different technologies, the Biomark system from Fluidigm (Fluidigm Corporation, South San Francisco, CA) has been recently demonstrated to have good analytical sensitivity and to be highly reproducible [13]. In this study, we assessed the dPCR methodology to detect and quantify residual and rare *BCR-ABL1* copies in *BCR-ABL1* positive ALL patients, and we compared obtained molecular results with those generated by conventional qPCR using ABI PRISM 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA).

Patients and Methods

60 *BCR-ABL1*-positive ALL samples in hematologic and cytogenetic remission (42 positive for the p190 *BCR-ABL1* isoform and 18 for the p210) were analyzed. Total cellular RNA was extracted from cells using the RNeasy total RNA isolation kit (QIAGEN, Valencia, CA) according to the instructions of the manufacturer and 1 µg was used for cDNA synthesis in the reverse transcriptase reaction (RT), as previously described [14]. For real time PCR analysis we used 5 µL of cDNA (corresponding to 100 ng of total RNA). TaqMan absolute quantitative PCR was performed on the ABI PRISM 7900HT Fast Real-Time PCR System (Applied Biosystems) and according to guidelines from the European Concerted Action [15-16]. RNA integrity was evaluated using the control gene *ABL1*. Both *BCR-ABL1* or *ABL1* copy number copies were derived by the interpolation of cycle threshold (Ct) values to the

appropriate standard curve obtained using different plasmid dilutions (Ipsogen, Marseilles, France), each containing known *BCR-ABL1* (10^6, 10^5, 10^3, 10^2, 10^1) or *ABL1* (10^5, 10^4, 10^3) gene copies. All real time RT-PCR experiments were performed in duplicate. The threshold was systematically set at 0.1 in order to avoid any particular problems of baseline creeping and results were expressed as a ratio of *BCR-ABL1* mRNA copies to *ABL1* mRNA copies per cent. Subsequently, we quantified the same leukemia cDNA samples using the 12.765 Digital array (Fluidigm). This is a nanofluidic biochip that consists in twelve panels, each containing 765 individual reaction chambers of 6 nL volume. Briefly, samples are portioned prior to qPCR into the single chambers of the panel; as fluorescent signal is produced only in chambers containing copies of the target sequence, digital array provides an absolute quantification by counting the number of positive reactions. Following amplification, digital raw data are processed by the BioMark Digital PCR Analysis software (Fluidigm) that estimates the true number of molecules per chamber using the Poisson probabilistic distribution. To minimise the uncertainty from pipetting, all components including Taqman Gene Expression MasterMix (Applied Biosystems), DA Sample loading reagent (Fluidigm), primers and probe were pre-mixed and then the final reaction mix was prepared by combining 2 µL cDNA solutions and the pre-mixed solution in a final volume of 8 µL. Subsequently the reaction mix was dispensed into each sample inlet and approximately 4.6 µL of this reaction was distributed throughout the partitions within each panel using an automated NanoFlex IFC Controller (Fluidigm) (the estimated amount of cDNA loaded in each panel is 1.15 µL). The digital array thermocycling conditions on the Bio-Mark System PCR (Default-10-min hotstar) consisted of a 10 min activation step at 95 °C, followed by 40 cycles of a two-step thermal profile involving 15 s at 95 °C for denaturation, and 60 s at 60 °C, for annealing and extension. Unlike real time RT-PCR, digital PCR does not collect data during the exponential phase of PCR, allowing reduction of PCR cycles. To ensure that cycle's number decrease does not affect data collection, two chips were run

twice with both 40 and 50 cycles. Standard deviation (SD), paired t-test, Pearson's correlation, variance ratio test and linear regression (confidence interval 95%) were calculated using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

Results and Discussion

Sixty samples from ALL patients in complete hematological and cytogenetic response were firstly analyzed by RQ-PCR with conventional method based on TaqMan chemistry and ABI PRISM 7900HT Fast Real-Time PCR System technology (Applied Biosystems). PCR results were expressed as *BCR-ABL1*/*ABL1* ratio% and they ranged between 0 and 0.39 (median 0.01). More in details, analyzed samples showed a ratio ≤ 0.001 in 36.67% (22/60), ≤ 0.01 in 18.33% (11/60), ≤ 0.1 in 36.67% (22/60) and >0.1 in 8.33% (5/60) (Tables 1-2). Then, we assessed the sensitivity and reproducibility of the Fluidigm assay using six serial dilutions of plasmids (Ipsogen) expressing known copy number of *BCR-ABL1* p190 transcript (10,000; 1,000; 100; 50; 10; 1 copies/2.5 µL) and two panels for each dilution were used in order to assess the reproducibility. Since the estimated amount of input DNA loaded is 1.15 µL the expected number of p190 transcript copies were 4600.00, 460.00, 46.00, 23.00, 4.60 and 0.46 for each dilution. The analysis of digital raw data was performed keeping the automated set threshold with Ct values ranging between 20 and 40 cycles. The assay detected until a copy of the target sequence (Figure 1) with a good concordance between replicates (paired t-test, p = ns) (Table 1S). The comparison between the estimated number of p190 transcript copies and those detected by Fluidigm PCR resulted in a standard deviation below 5.6 until 46 expected copies (Table 2S). This value defines a detection limit needed to maintain a high sensitivity, over which a dilution of input cDNA is recommended. Nevertheless, copies detected by Fluidigm correlated to those estimated according to a linear

relationship (Pearson's correlation coefficient 0.9996, p<0.0001). We then analyzed *BCR-ABL1* positive leukemia cDNA samples previously quantified by ABI PRISM 7900 instrument. For each sample, we performed two independent quantifications using Fluidigm platform. A positive control was used for each chip. Digital array results were positive (at least one copy) in 22 samples (36.67%; median number of detected copies: 14.13, range 2.17-145.65/100 ng total RNA). In order to estimate the relation between the two measurements, we calculated the correlation coefficient. As well as for plasmids quantification, Fluidigm copies correlate to those obtained by *BCR-ABL1* quantification using ABI PRISM 7900 (Pearson's correlation coefficient 0.8232, p<0.0001), confirming the reliability of this approach. Interestingly, we found a linear correlation also between Fluidigm results and *BCR-ABL1/ABL1* ratio %, which is the current way to report results (p<0.0001). Subsequently, we compared Fluidigm and ABI PRISM 7900 results in order to define the agreement between the *BCR-ABL1* measures obtained with the two different platforms. Firstly, we compared the variance between the two independent quantification sets by means of the variance ratio test (F-test). As illustrated in Table 2, we did not find any significant difference among the variances ($F = 1.20 \times 10^{-12}$), showing the precision of measurements and the agreement of the two methods. Then, we evaluated the discrepancies in *BCR-ABL1* copies detection by plotting the difference between the methods against their means[17]. As shown in Fig. 2 most of the differences lie between mean ± 2 SD values (-28.0 and 38.94 *BCR-AB1* copies) accordingly to a Gaussian distribution. These values are defined as "limits of agreement", and indicate the range of differences in *BCR-ABL1* detection that might exist among Fluidigm and ABI PRISM 7900 methods. Of note, the plot highlighted the scattering of the differences corresponding to increased values of *BCR-ABL1* copies. Partition of the measurements into subgroups (average *BCR-ABL1* copies < 10 or average *BCR-ABL1* copies <1) led to the reduction of the limits of agreement (-8.06 and 8.43; -0.91 and 1.67, respectively). These values delineated a range that is likely more acceptable for clinical

purposes, enforcing Fluidigm robustness in detection of rare copies of genes and confirming the requirement of diluted input material to maintain high sensitivity (as previously evaluated through plasmid analysis). In conclusion, we demonstrated that dPCR by Fluidigm nanofluidic platform provides a high sensitive assay, able to detect until a single copy of *BCR-ABL1* transcript, as demonstrated by plasmid serial dilution detection rate and by quantification of samples in molecular remission. Therefore, it could provide an accurate monitoring method for *BCR-ABL1*-positive ALL patients in complete remission. Digital PCR is an alternative technique for quantifying gene copy number which may provide more accurate measurements than other approaches currently available as it is not dependant on amplification efficiency. One of the main advantages of this method is the possibility to exploit the TaqMan chemistry that guarantees a high specific detection rate due to annealing between probes and target genes. Moreover, Fluidigm digital chips provide a direct and absolute quantification of gene expression related only to the RNA amount. Therefore, using this approach is possible to quantify and compare gene transcript levels independently to the cellular context (e.g. cellular stress, drug's effects, microenvironment effects) or despite the presence of a common and invariant housekeeping gene to which refer the results, as in relative quantification. Fluidigm digital chips have also proven to reach a high sensitivity, therefore by using appropriate cDNA dilutions is possible to quantify both rare or common and highly expressed genes. Finally, the application of different fluorocrome-labeled probes allows simultaneous quantification of different transcript in the same sample, with important implication when transcript amount comparison is required [18]. Based on the advantages of dPCR by Fluidigm respect to conventional quantification by ABI PRISM 7900, we believe that this approach may provide an accurate measurement of MRD in ALL patients in complete hematological and cytogenetic remission. Application of this approach in prospective samples enrolled in clinical trials will shed light on its role in predicting relapse.

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Tables

Table 1. *BCR-ABL1/ABL1* ratio % obtained by RQ-PCR with ABI PRISM 7900 technology in 60 ALL samples.

***BCR-ABL1/ABL1* %**	**≤ 0.001**	**≤ 0.01**	**≤ 0.1**	**> 0.1**
Samples				
Tot (n=60)	22 (36.67%)	11 (18.33%)	22 (36.67%)	5 (8.33%)
p190 (n=42)	19 (31.66%)	6 (10.00%)	13 (21.66%)	4 (6.66%)
p210 (n=18)	3 (5.00%)	5 (8.33%)	9 (15.00%)	1 (1.66%)

Table 2. Comparison between ABI PRISM 7900 (ABI) and Fluidigm (FL) quantification in leukemia samples.

ID	Ratio *BCR-ABL1/ABL1* % ABI	*BCR-ABL1* copies/ 100 ng RNA ABI	*BCR-ABL1* copies/ 100 ng RNA FL	Average *BCR-ABL1* copies	Difference in *BCR-ABL1* copies (FL – ABI)
#12	0	**0**	**0**	0.00	0.00
#14	0	**0**	**0**	0.00	0.00
#15	0	**0**	**0**	0.00	0.00
#17	0	**0**	**0**	0.00	0.00
#18	0	**0**	**0**	0.00	0.00
#21	0	**0**	**0**	0.00	0.00
#22	0	**0**	**0**	0.00	0.00
#28	0	**0**	**0**	0.00	0.00
#31	0	**0**	**0**	0.00	0.00
#34	0	**0**	**0**	0.00	0.00
#35	0	**0**	**0**	0.00	0.00
#36	0	**0**	**0**	0.00	0.00
#38	0	**0**	**0**	0.00	0.00
#46	0	**0**	**0**	0.00	0.00
#47	0	**0**	**0**	0.00	0.00
#49	0	**0**	**0**	0.00	0.00
#51	0	**0**	**0**	0.00	0.00
#58	0	**0**	**0**	0.00	0.00
#60	0	**0**	**0**	0.00	0.00
#16	0.001	**0.253**	**0**	0.13	-0.25
#26	0.001	**0.326**	**0**	0.16	-0.33
#45	0.007	**0.424**	**0**	0.21	-0.42
#56	0.012	**0.642**	**0**	0.32	-0.64
#27	0.016	**1.002**	**0**	0.50	-1.00
#13	0.009	**1.364**	**0**	0.68	-1.36
#20	0.042	**1.538**	**0**	0.77	-1.54
#37	0.052	**1.577**	**0**	0.79	-1.58
#24	0.005	**1.891**	**0**	0.95	-1.89
#33	0.032	**1.918**	**0**	0.96	-1.92
#32	0.010	**1.994**	**0**	1.00	-1.99
#59	0.086	**2.056**	**0**	1.03	-2.06
#53	0.060	**2.062**	**0**	1.03	-2.06
#39	0.037	**2.546**	**0**	1.27	-2.55
#25	0.005	**2.733**	**0**	1.37	-2.73
#5	0.009	**0.658**	**2.174**	1.42	1.52
#30	0.029	**4.102**	**0**	2.05	-4.10
#55	0.001	**0.020**	**4.348**	2.18	4.33

#7	0.007	**0.957**	**4.348**	2.65	3.39
#57	0.025	**1.190**	**4.348**	2.77	3.16
#52	0.052	**1.577**	**4.348**	2.96	2.77
#41	0.106	**1.818**	**4.348**	3.08	2.53
#54	0.062	**3.146**	**4.348**	3.75	1.20
#4	0.028	**1.680**	**6.522**	4.10	4.84
#23	0.028	**9.283**	**0**	4.64	-9.28
#43	0.390	**9.582**	**0**	4.79	-9.58
#42	0.010	**0.679**	**10.870**	5.77	10.19
#29	0.035	**15.346**	**0**	7.67	-15.35
#1	0.035	**2.889**	**13.043**	7.97	10.15
#44	0.144	**5.533**	**13.043**	9.29	7.51
#3	0.013	**0.377**	**26.087**	13.23	25.71
#2	0.038	**12.481**	**15.217**	13.85	2.74
#10	0.015	**2.672**	**28.261**	15.47	25.59
#48	0.362	**10.078**	**21.739**	15.91	11.66
#40	0.067	**6.119**	**26.087**	16.10	19.97
#50	0.342	**8.589**	**30.435**	19.51	21.85
#8	0.015	**2.339**	**41.304**	21.82	38.97
#9	0.020	**5.737**	**41.304**	23.52	35.57
#6	0.143	**26.340**	**23.913**	25.13	-2.43
#11	0.298	**15.251**	**82.609**	48.93	67.36
#19	0.226	**57.080**	**145.652**	101.37	88.57
Std. Dev.		**8.504**	**23.037**		
Mean		**3.798**	**9.239**		

Figure legend

Figure 1. Amplification plot (left) and panel redouts (right) of dPCR reaction of *BCR-ABL1* copies contained in plasmid dilutions with 4600, 460, 46, 23, 4.6 and 0.46 estimated copies. Chambers containing target sequence are positive for fluorescence signal detection, and appear red coloured. Grey colour signifies partitions with no amplification.

Figure 2. Scattering of BCR-ABL1 copies as determined by ABI PRISM 7900 and Fluidigm.

Conflict of interest

The authors have no conflicts of interest.

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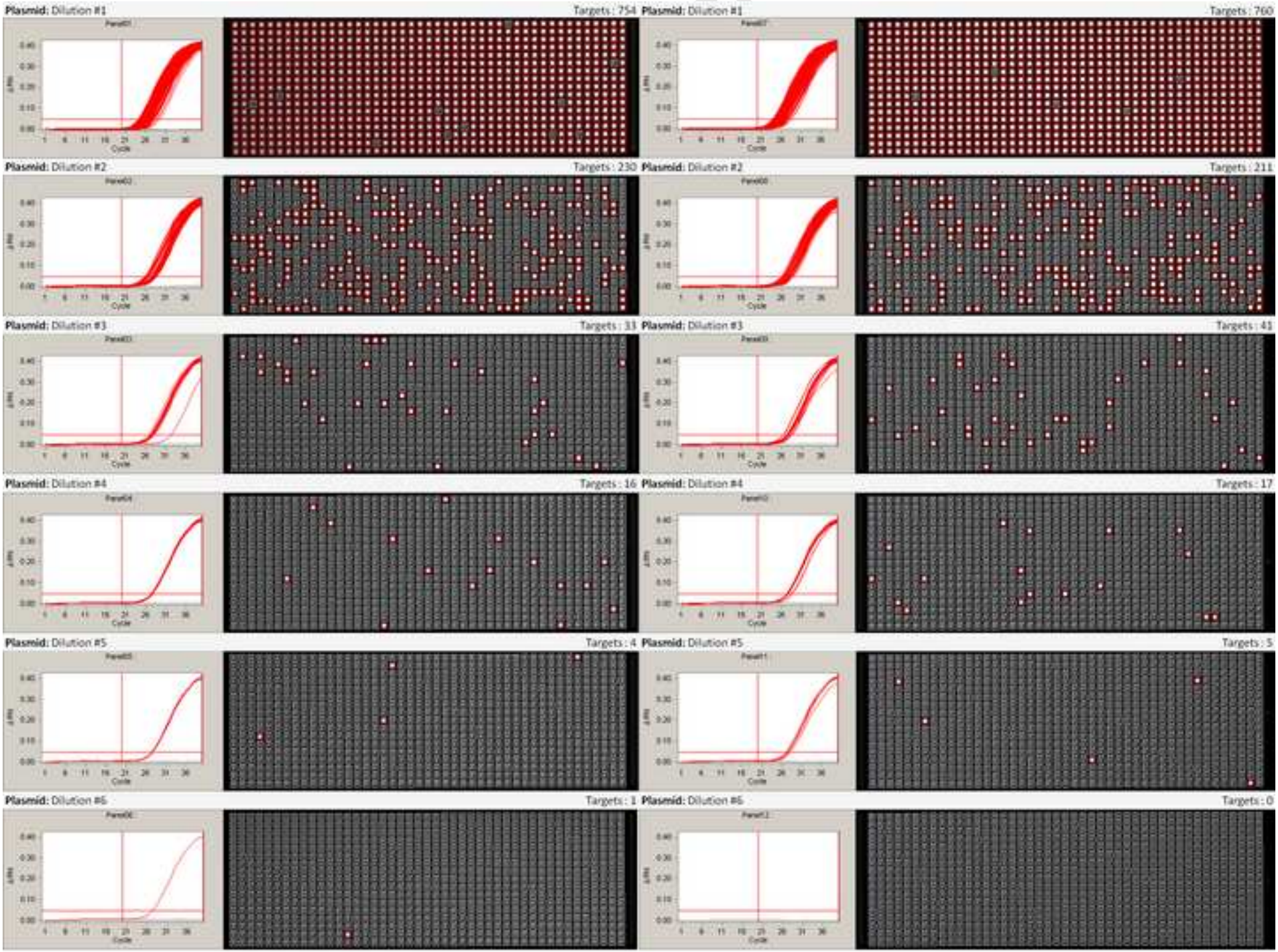
Final approval of manuscript: Ilaria Iacobucci, Giovanni Martinelli

Tables legend

Table 1. *BCR-ABL1/ABL1* ratio % obtained by RQ-PCR with ABI PRISM 7900 technology in 60 ALL samples.

Table 2. Comparison between ABI PRISM 7900 (ABI) and Fluidigm (FL) quantification in leukemia samples.

Figure 1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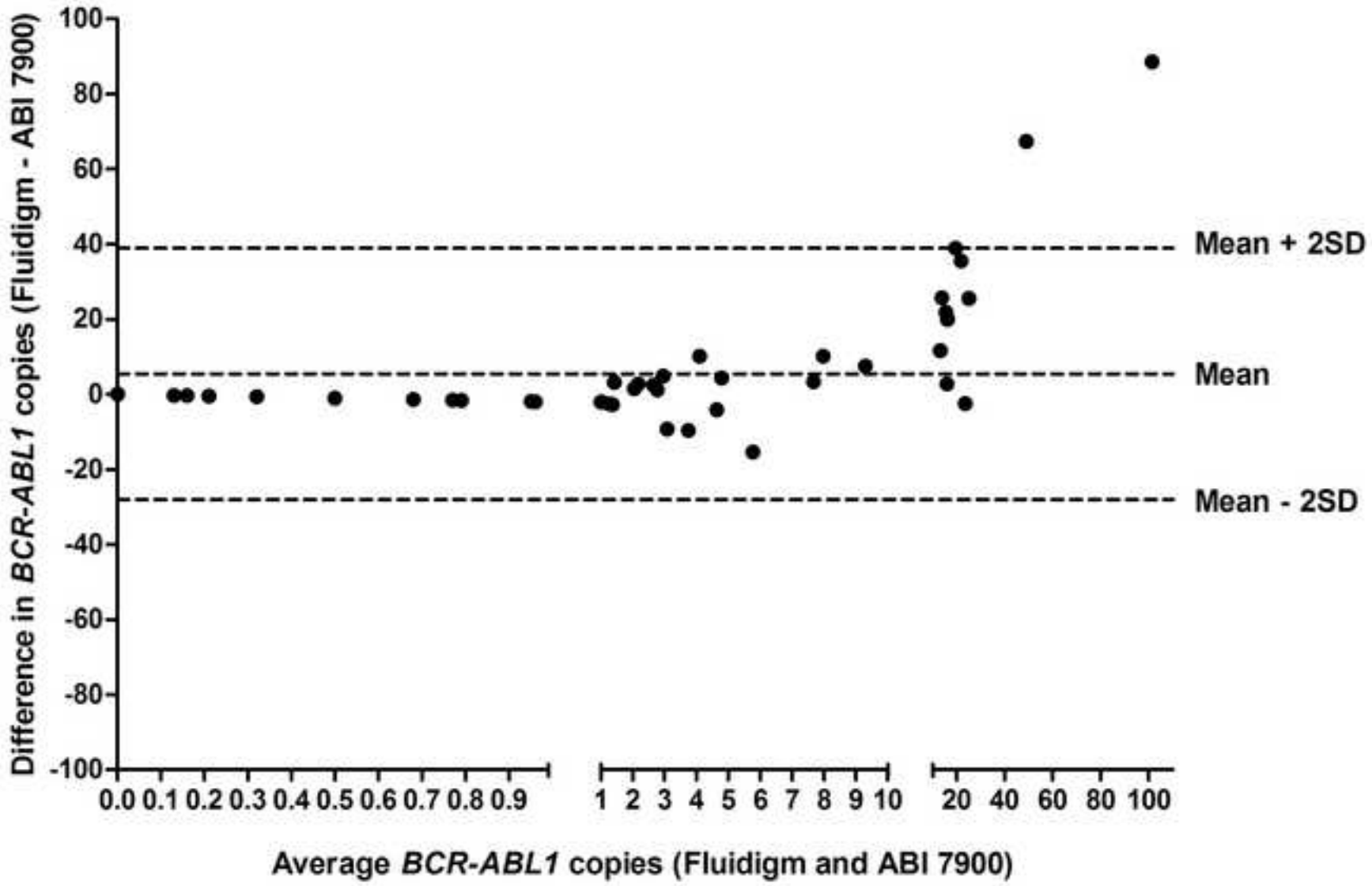
Targets : 754
Plasmid: Dilution #1
Targets : 760
Plasmid: Dilution #2
Targets : 230
Plasmid: Dilution #2
Targets : 211
Plasmid: Dilution #3
Targets : 33
Plasmid: Dilution #3
Targets : 41
Plasmid: Dilution #4
Targets : 16
Plasmid: Dilution #4
Targets : 17
Plasmid: Dilution #5
Targets : 4
Plasmid: Dilution #5
Targets : 5
Plasmid: Dilution #6
Targets : 1
Plasmid: Dilution #6
Targets : 0

Figure 2


Difference in BCR-ABL1 copies (Fluidigm - ABI 7900)
100
80
60
40
20
0
-20
-40
-60
-80
-100
Mean + 2SD
Mean
Mean - 2SD
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9
1 2 3 4 5 6 7 8 9 10
20 40 60 80 100
Average BCR-ABL1 copies (Fluidigm and ABI 7900)