Development of highly sensitive detection for *BCR-ABL* transcripts in chronic myeloid leukemia by droplet digital PCR

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ABSTRACT

BCR-ABL tyrosine kinase inhibitors (TKIs) have been the standard treatment for chronic myeloid leukemia (CML). Imatinib is the first-line standard therapy for CML and molecular response assessment of minimal residual disease (MRD) is based on the quantitation of *BCR-ABL* transcripts using real-time quantitative polymerase chain reaction (RQ-PCR). More sensitive assays for MRD are required to identify patients who are more likely to remain in remission without therapy. In order to improve the performance of conventional RQ-PCR, it is necessary to consider the factors that limit the detection and specificity of the method. However, droplet digital PCR (ddPCR) approaches this problem in a different way. Using nanofluidic technology, the nucleic acid sample is divided into hundreds of individual replicate PCR reactions. Using *BCR-ABL* p210 plasmid and *BCR-ABL* transcripts from patient sample dilutions, we demonstrated that ddPCR is a highly sensitive method with lower limit of detection, compared with the conventional RQ-PCR method. The results from ddPCR showed successful detection of *BCR-ABL* transcripts in samples undetectable by RQ-PCR, and low sensitivity limit by detecting as few as 10 copies per microliter. The correlation of measurements between RQ-PCR and ddPCR from plasmid and clinical samples indicated remarkable correlation between both techniques. Moreover, ddPCR was able to detect *BCR-ABL* transcripts in specimens from one CML patient who sustained CMR by conventional RQ-PCR before relapsed. This approach results in more efficient amplification of *BCR-ABL* transcripts that may provide an accurate measurement of MRD in CML patients with CMR. Application of this approach in prospective samples enrolled in clinical trials will shed light on its role in predicting relapse. We anticipate that the method will be employed in multiple applications in the clinic, including diagnosis, cancer recurrence monitoring, and treatment management.

Keywords: *BCR-ABL* transcripts; complete molecular response (CMR); conventional RQ-PCR; droplet digital PCR (ddPCR); minimal residual disease (MRD)

INTRODUCTION

Chronic myeloid leukemia (CML) is a proliferative disease originating in a pluripotent hematopoietic stem cell that has acquired a Philadelphia (Ph) chromosome encoding the *BCR-ABL* oncogenic fusion protein (Rowley, 1973; Groffen *et al*., 1984). Treatment

strategies based on tyrosine kinase inhibitors (TKIs) of the first and second generation drug have substantially improved overall treatment results, with rapid remission and patients will achieve complete molecular response (CMR) rates in estimated 40%, after 5 years on imatinib treatment, as defined by conventional RQ-PCR (Branford *et al*., 2007). However, from the studies on the discontinuation of TKI therapy, 100% of patients with less than CMR have relapsed (Goh *et al*., 2009; Kuwabara *et al*., 2010) and over 50% of patients who stop imatinib after a period of stable CMR will relapse within 6 months, whereas nearly all of the remaining patients sustain stable CMR with follow-up beyond 18 months (Rousselot *et al*., 2007).

Molecular response is the measurement of *BCR-ABL* transcripts and described as a ratio of *BCR-ABL* to a housekeeping gene (such as *ABL* or *BCR*). The international scale (IS) has been developed to allow cross-laboratory comparison of PCR results as defined by a baseline value of the *BCR-ABL/ABL* ratio. This ratio is based on an analysis of baseline levels in patients from the IRIS trial (100%), or a 3-log reduction thereof, or a reduction to 0.1% or less, which is defined as an MMR (Hughes *et al*., 2003; Branford *et al*., 2008). It was reported that RQ-PCR method could detect deeper MRD levels of response, up to a 5-log reduction (Kantarjian *et al*., 2008). However, in routine laboratory, an estimated detection limit of 4.5 logs below the median pretreatment *BCR-ABL* value (standardized baseline) was used in the IRIS study to define samples as having no detectable *BCR-ABL* transcripts (Branford *et al*., 2007).

Due to the fact that the absolute number of residual leukemia clones could not be measured under the detection limits of conventional RQ-PCR detection (4.5- log reduction), it might have resulted in relapse in more than half of the patients. This indicates that conclusions cannot be drawn whether a patient could safely discontinue therapy solely based on the conventional RQ-PCR. The absence of *BCR-ABL* transcripts in the conventional RQ-PCR is sometimes unreliable since some patients, despite being PCR-negative, relapse and PCR negativity may be maintained after imatinib discontinuation (Mauro *et al*., 2004). Therefore, more sensitive PCR methods are required to detect minimal residual disease (MRD). It can provide more reliable estimation of the therapeutic response of the patient, and thus allow further classification of patients who may discontinue imatinib without the risk of relapse.

Since then many studies demonstrated the improvement of limit of detection such as modification reverse transcription methodology with random pentadecamer primers (Ross *et al*., 2008), detection of genomic *BCR-ABL* DNA using a highly sensitive patient-specific nested quantitative PCR (Ross *et al*., 2010), increasing the number of well from 2 wells (duplicated) to 90 wells (replicated) in conventional RQ-PCR and detecting *BCR-ABL* transcripts by digital polymerase chain reaction (dPCR) (Goh *et al*., 2011).

Digital PCR as a third generation of PCR technology is a method of absolute nucleic acid quantification based on the partitioning nanoliter or even picoliter scale by creating reaction chambers within specially designed chips or sequestered reagents that became several individual droplets. Finally, digital PCR generates many replication reactions and can do direct counting of positive and negative reactions of the target molecules (Monya *et al*., 2012). The digital PCR has many advantages over real-time PCR, including the capability to obtain absolute quantification without external references and its robustness to identify variations in PCR efficiency (Bustin *et al*., 2004). The practical dynamic range of the system is substantially improved by using highly uniform droplets during Poisson

statistics correction for multiple target molecules per droplet or chamber to enable the precise calculation of concentrations even above conditions of limiting dilution (Hindson *et al*., 2011; Pinheiro *et al*., 2012). One application of digital PCR is to detect minimal residual disease molecule since it has already been proved that the template molecules are partitioned throughout the panels with a high degree of randomness and independence (Bhat *et al*., 2009). The increase in target-to-background ratio theoretically leads to 765-fold improvement in detection sensitivity by biochip (Goh *et al*., 2011). Moreover, detection by droplet PCR technique increases target-to-background ratio and leads to 20,000-fold more sensitive (Hindson *et al*., 2011). Thus sample partitioning eventually permits detection of very low levels of MRD with the lessened impact of dilution by background molecules (Goh *et al*., 2011).

In this study, a new molecular PCR technology approach based on droplet digital polymerase chain reaction (ddPCR) using Taqman chemistry will be used to compare the results with those generated by the conventional method RQ-PCR for *BCR-ABL* transcript measurement. The ddPCR technology is applied to test capability limits of *BCR-ABL* transcript quantification in p210 plasmid standard and CML patients. Subsequently, we evaluated undetectable *BCR-ABL* sample in a CMR patient. The results demonstrated that ddPCR is highly sensitive enough to detect a very low copy of *BCR-ABL* transcripts. The advantages of ddPCR with regard to lower limit leads to an accurate monitoring of *BCR-ABL* CML patients in CMR status.

MATERIALS AND METHODS

Plasmid DNA dilutions

A ten-fold serial dilution was conducted in plasmid DNA of p210 *BCR-ABL* and used to prepare standard curve in conventional RQ-PCR assay.

Patient samples

A CML patient with undetected minimal residual disease by RQ-PCR in minimum of 2 consecutive visits was included in the study. Three blood RNA samples were extracted using QIAamp RNA Blood Mini Kit (Qiagen, Valencia, CA, USA) from this patient and 100 ng of RNA were reverse transcribed using a TranscriptorSuperScript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). cDNA was used to test the sensitivity of ddPCR assay. The research proposal of this project was approved by Ramathibodi Hospital Ethic Committee on Human Rights Related to Researches Involving Human Subjects (ID 03-56-16).

BCR-ABL primer and probe design

The *BCR-ABL* primer and probe set designed by the EAC (Europe Against Cancer) group was chosen (Gabert *et al*., 2003). The forward primer ENF501, located on *BCR* exon 13 (exon b2), was used to amplify both *BCR-ABL* b3-a2 (e14a2) and b2-a2 (e13a2). *BCR-ABL* fusion transcripts (b3a2 and b2a2) were detected by TaqMan TAMRA probes and primers (Applied Biosystems, CA, USA).

Conventional real-time quantitative polymerase chain reaction (RQ-PCR)

Quantitative *BCR-ABL* and *ABL* transcripts testing were performed using TaqMan Probe on a conventional PCR machine (ABI PRISM 7900HTFast Real-Time PCR System; Applied Biosystems, CA, USA). All the RQ-PCR using primers and TaqMan probes provided by Applied Biosystems in conjunction with the TaqMan Universal Master Mix purchased from the same manufacturer. The number of amplification cycles was 50 from 95°C for 15 s, 60°C for 1 min with template started in 5 µl of final cDNA (100 ng RNA equivalent) following Standardized EAC RQ-PCR guidelines (Gabert *et al*.,

2003; Hughes *et al*., 2006). A seven-point standard curve (10^8 down to 10^2 gene copies) as well as a positive and a negative controls were included on all runs. The results were calculated as a ratio of *BCR-ABL* mRNA copies to *ABL* mRNA copies, expressed in percent. The ratio is converted to the international scale by multiplying with the conversion factor (Gabert *et al*., 2003).

Droplet digital PCR amplification

Serial dilutions of standards for *BCR-ABL* transcripts and leukemia cDNA samples were quantified using the QX100™ Droplet Digital PCR system (Bio-rad, Hercules, CA, USA). The ddPCR reaction mixture consisted of 10 µl of a 2x ddPCR supermix (Bio-Rad), 1.5 µl of *BCR-ABL* 750 nM primer and 450 nM probe mix, and 5 µl of cDNA sample solution in a final volume of 20 µl. The entire reaction mixture was loaded into a disposable plastic cartridge (Bio-Rad) together with 70 µl of droplet generation oil (Bio-Rad) and placed in the droplet generator (Bio-Rad). After processing, the droplets generated from each sample were transferred to a 96-well PCR plate (Eppendorf, Germany). PCR amplification was carried out on the 96-well C1000 Touch Thermal cycler (Bio-rad) using a thermal profile of beginning at 95°C for 10 min, followed by 50 cycles of 95°C for 15 s (ramp rate 2.5 °C/second) and 60°C for 60 s, 1 cycle of 98°C for 10 min, and ending at 12°C. After amplification, the plate was loaded on the droplet reader (Bio-Rad) and the droplets from each well of the plate were read automatically at a rate of 32 wells per hour. The droplets were subsequently read the positive from one-color fluorescence in FAM signal to determine many droplets of the *BCR-ABL* fusion transcript by the QX100™ droplet reader (Bio-Rad) and the data was analyzed with the QuantaSoft™ analysis software 1.3.2.0 (Bio-Rad) and the quantification of the target molecule was presented as the number of copies per µl of PCR mixture. The ddPCR workflow was shown in Figure 1.

Statistical analysis

Linear regression test was used to determine a linear relationship between relative RQ-PCR and absolute ddPCR in plasmid standard and clinical sample dilutions with 95% confidence intervals (CI). Statistical test was performed with data analysis excel 2007 software.

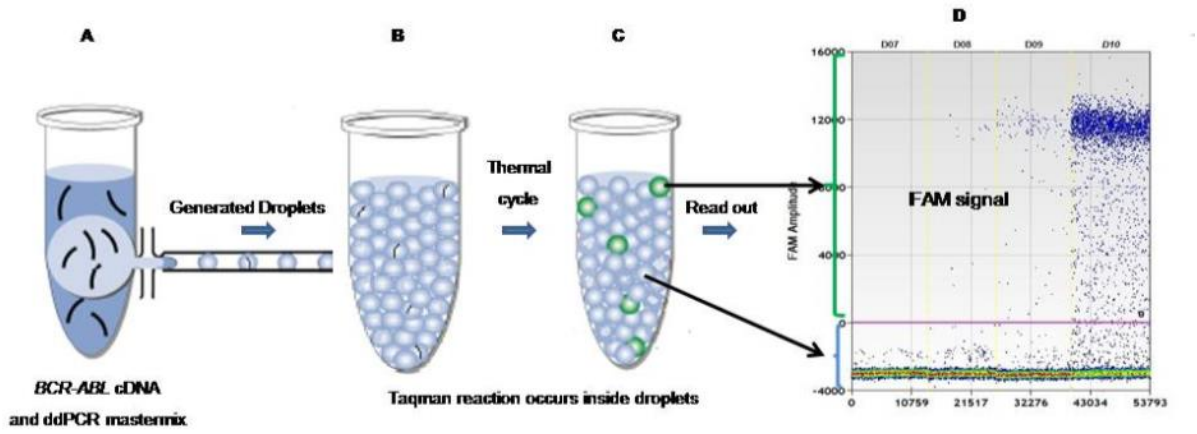

Figure 1 Droplet digital PCR approach. A) Patient sample containing low level of cancer sequences in a high background of non-specific sequences. B) Sample diluted to single molecule per droplet and partitioned into millions of reaction droplets. C) End point PCR produces positive and negative reactions. D) Droplets flow past laser; one color fluorescent detection yields positive droplets.

RESULTS

Schematic representation of ddPCR for quantitative *BCR-ABL* transcripts is shown in Figure 1. We analyzed the sensitivity of ddPCR assay using p210 plasmid serial dilution (10^5, 10^4, 10^3, 10^2, 10, and 10^0 copies/µl) and patient sample dilutions of known *BCR-ABL* copy numbers (8,000, 800, 80, 8, 0.8 copies/µl). The limit of detection (LOD) was as low as 10 and 8 copies/µl in plasmid and patient sample dilutions, respectively (Figures 2A and 2B). In plasmid and sample dilutions, *BCR-ABL* quantitation by ddPCR correlated with the *BCR-ABL* levels measured by RQ-PCR after converting to log-log scale, linear regression showed R^2 = 0.999965 and 0.999932, respectively (Figures 3A and 3B). In addition, all negative samples showed negative result in every panel of ddPCR.

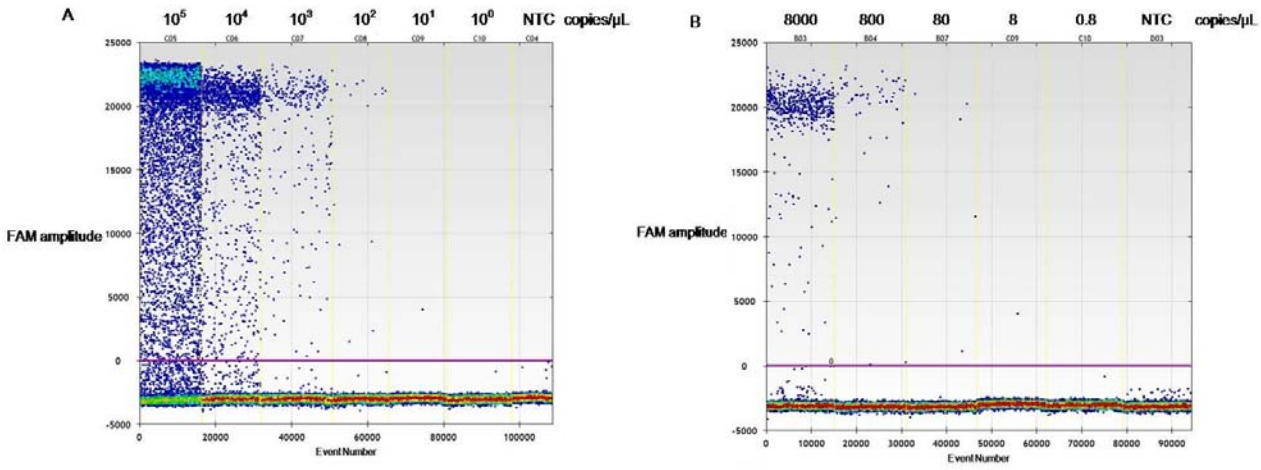

Figure 2 FAM fluorescence amplitudes of plasmid and sample dilution: (A) p210 plasmid dilutions expressing known *BCR-ABL* copy numbers (10^5–10^0 copies/µl) and one NTC (non-template control) in Channel no. C05-C10 and C04, respectively. (B) Patient sample dilutions expressing *BCR-ABL* transcripts of 8,000, 800, 80, 8, 0.8 copies/µL and one NTC (non-template control) in Channel no. B03, 04, 07, C09-10 and D03, respectively.

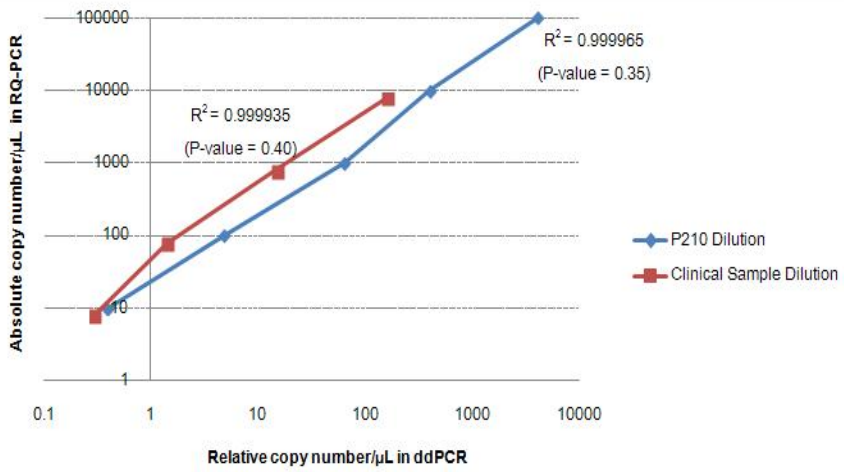

Figure 3 Log-log plot between absolute copy number by RQ-PCR and relative copy number by ddPCR. p210 plasmid dilutions (R^2=0.999965, *p*-value=0.35) and clinical sample dilutions (R^2=0.999935, *p*-value=0.40) were shown.

To assess possible false positive droplets for the ddPCR run, non-template controls (NTCs) with DEPC-treated water were included in every run and a total of six normal controls from normal clinical sample cDNA were tested. Results showed that all of normal control samples were entirely negative and NTCs remained negative in every panel (Figure 4). Consequently, the ddPCR assay was applied to three samples from one CML patient before relapse that was undetectable by RQ-PCR. The three samples were obtained from different follow up periods of this patient. The results of ddPCR and RQ-PCR were shown in Table 1. *BCR-ABL* transcripts from 1 of 3 samples were detected up to 54.8 copy number by ddPCR (Table 1 and Figure 5).

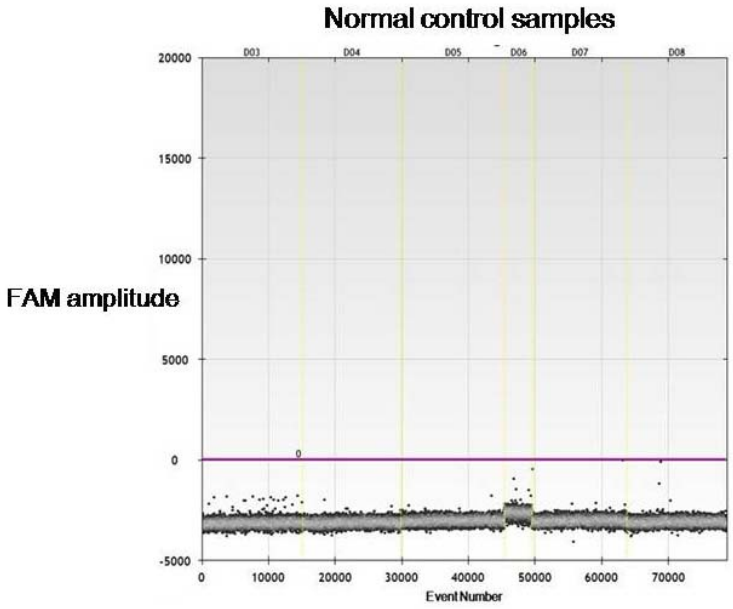

Figure 4 Representative results of normal control samples in channel no. D03–08.

Table 1 *BCR-ABL* transcript detection in 6 follow up samples of one CML patient.

Time	Date	Tube ID	% BCR-ABL/ABL (IS*)	BCR-ABL Copy Number By RQ-PCR	BCR-ABL Copy Number By ddPCR	ABL Copy Number By RQ-PCR	BCR-ABL/ABL Ratio
1	08/02/55	55RQ0148	32.35	143,220.00	NA	70,837.60	2.02
2	16/05/55	55RQ0437	2.75	11,438.10	NA	66,516.20	0.17
3	08/08/55	55RQ0745	-	Undetected	54.80	276,026.00	-
4	20/02/56	56RQ0179	-	Undetected	1.45	7,622.42	-
5	28/08/56	56RQ1000	-	Undetected	2.98	35,317.60	-
6	01/08/57	57RQ0029	0.012	17.94	NA	23,076.70	0.00078

IS = international standard, NA = Not available

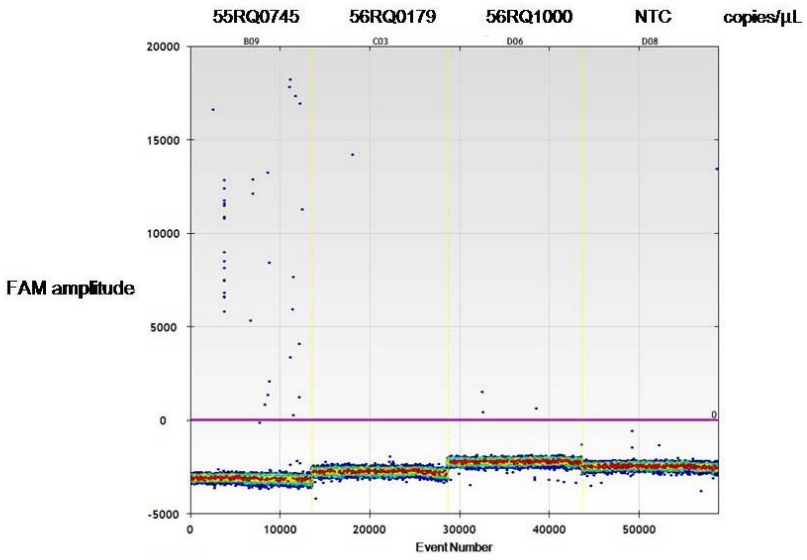

Figure 5 Three samples from a patient were detected for *BCR-ABL* transcripts by ddPCR. Tube ID 55RQ0745, which was obtained from the earlier visit compared to the 56RQ0179 and 56RQ1000, was detected to have many positive droplets.

DISCUSSION

While *BCR-ABL* inhibition or imatinib became to failure for treatment, it would be of clinical value to begin second-line TKI therapeutics (Baccarani *et al*., 2013). To achieve deeper response at the molecular level, more sensitive quantitation method is necessary to be established for detection of the minimal residue disease (MRD). RQ-PCR, which is a popular method to detect MRD, can detect deeper up to 4.5 log reduction while MRD level below 4.5 log-limit of detection cannot be detected by RQ-PCR (Mauro *et al*., 2004; Branford *et al*., 2007). We aimed to establish a high sensitive method to detect MRD in CML patients who achieved CMR.

In our study, we demonstrated that ddPCR enables accurate and precise measurements of fusion gene over traditional RQ-PCR assay. Our data showed R^2 to be nearly 1. However, the absolute cDNA copy numbers quantified by ddPCR were lower than the corresponding cDNA copy number assessed by RQ-PCR. One explanation is that a higher copy number value by RQ-PCR can be influenced by factor in which relative to the standard curve which related to the amount of clone of plasmid that measured on UV-spectrophotometry and calculated by formula. In practice, the interpretation of RQ-PCR results near the limit of detection is not so simple. In our laboratory, for instance, the lower limit of detection is set at a threshold cycle number (Ct) equivalent to 100 copies of *BCR–ABL* which is determined from the standard curve. RQ-PCR is an analog system: the result (Ct) is a continuous variable; thus, the cut-off was determined empirically as giving the best distinction between true low positive results and the low level of amplification that may be seen in negative control samples.

In a previous study, ddPCR was used to quantitate the total number of amplification targets and the increased partitioning further increased the limit of detection and quantification as international scale (IS) (Jennings *et al*., 2014). However, that study used known %IS pooled clinical sample detected by RQ-PCR compared to IS calibrators

panels; hence, the results was a ratio of *BCR-ABL* copies to internal control copies, which does not represent the real %IS units. In addition, ddPCR does not require calibration and internal control. Thus, in our study we did not use standard curve and internal control (neither the *BCR* nor the *ABL* genes). Therefore, data shown in this study refer to copy number per microliter instead of the international scale (IS) unit that defined by a baseline value of *BCR-ABL* per internal control ratio and convert PCR result with a laboratory specific conversion factor. In the case of RQ-PCR, the raw data-to-cDNA conversion factors were calculated based on the standard curve. This conversion factor was used in the patient samples to convert Cq to cDNA copy number. Therefore, results of RQ-PCR as a relative copy number unlike absolute copy number that ddPCR directly detected at endpoints of the PCR reaction. Goh and their colleague demonstrated the use of digital PCR to detect *BCR-ABL* fusion transcripts which partitions each sample into 765 separate wells (Goh *et al*., 2011). In their study, digital PCR was used in conjunction with a pre-amplification step. However, from our impression, that method did not represent a real copy number of *BCR-ABL* transcripts in clinical sample because the pre-amplification step involves the expansion of the amount of original copy number which is not the amount of absolute original copy number in a sample. For our assay, the sample in a single well is partitioned into up to 20,000 droplets. The increased partitioning further increases the limits of detection and quantification; thus, pre-amplification is not necessary. Using ddPCR, we found MRD in three samples undetectable by RQ-PCR before relapse. The results suggested that the highly sensitive detection of *BCR-ABL* transcripts by ddPCR would identify patients with low level of residual leukemic clone that may not be detectable by the RQ-PCR technique.

In addition, several studies reported the comparison of ddPCR versus RQ-PCR performance including technical precision and reproducibility and revealed that ddPCR could improve greater precision and decrease variation especially lower copy targets. ddPCR has an advantage for detection of MRD and they described application of ddPCR such as the measurement of germline copy number variation, highly sensitive detection of mutant DNA in a 100 000-fold excess of wild type background and absolute quantitation of circulating fetal and maternal DNA from cell-free plasma (Hayden *et al*., 2013; Hindson *et al*., 2013; Jennings *et al*., 2014; Kiselinova *et al*., 2014). Moreover, another study in Thailand showed that ddPCR may be an alternative technology appropriate for routine clinical diagnosis of α(0)-thalassemia SEA-type deletion and prenatal diagnosis of Bart's hydrops fetalis (Pornprasert and Prasing, 2014). Therefore, we expect that the ddPCR system will allow researchers to explore complex genetic, validate new disease associations, and is applied in highly sensitive molecular diagnostics which RQ-PCR cannot performed.

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