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RT-qPCR and RT-Digital PCR: A Comparison of Different Platforms for the Evaluation of Residual Disease in Chronic Myeloid Leukemia

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BACKGROUND: Tyrosine kinase inhibitors (TKIs) are the cornerstone of successful clinical management of patients with chronic myeloid leukemia (CML). Quantitative monitoring of the percentage of the BCR, RhoGEF, and GTPase activating protein–ABL proto-oncogene 1, non-receptor tyrosine kinase fusion transcript *BCR-ABL1*^{IS} (%*BCR-ABL1*^{IS}) by reverse transcription–quantitative PCR (RT-qPCR) is the gold standard strategy for evaluating patient response to TKIs and classification into prognostic subgroups. However, this approach can be challenging to perform in a reproducible manner. Reverse-transcription digital PCR (RT-dPCR) is an adaptation of this method that could provide the robust and standardized workflow needed for truly standardized patient stratification.

METHODS: *BCR-ABL1* and *ABL1* transcript copy numbers were quantified in a total of 102 samples; 70 CML patients undergoing TKI therapy and 32 non-CML individuals. 3 commercially available digital PCR platforms (QS3D, QX200 and Raindrop) were compared with the platform routinely used in the clinic for RT-qPCR using the EAC (Europe Against Cancer) assay.

RESULTS: Measurements on all instruments correlated well when the %*BCR-ABL1*^{IS} was ≥0.1%. In patients with residual disease below this level, greater variations were measured both within and between instruments limiting comparable performance to a 4 log dynamic range.

CONCLUSIONS: RT-dPCR was able to quantify low-level *BCR-ABL1* transcript copies but was unable to improve sensitivity below the level of detection achieved by RT-qPCR. However, RT-dPCR was able to perform these sensitive measurements without use of a calibration curve. Adaptions to the protocol to increase the amount of RNA measured are likely to be necessary to improve the analytical sensitivity of *BCR-ABL* testing on a dPCR platform.

Tyrosine kinase inhibitors (TKIs)[9] allow successful clinical management of patients with chronic myeloid leukemia (CML) *(1)*. Quantitative monitoring of BCR, RhoGEF, and GTPase activating protein–ABL proto-oncogene 1, non-receptor tyrosine kinase (*BCR-ABL1*)[10] transcripts in peripheral blood by reverse transcription-quantitative PCR (RT-qPCR) is the gold standard strategy to evaluate response to TKI therapy. The log reduction of transcript levels at particular time points is prognostic and is used to stratify patients and to predict the risk of treatment failure *(2–6)*. This form of monitoring has been adopted into the ELN (European Leukemia Network) guidelines. Patients are classified as good responders when the percentage of *BCR-ABL1*^{IS} (%*BCR-ABL1*^{IS}) is ≤10% at 3 months after treatment initiation followed by ≤1% at 6 months and ≤0.1% at 12 months [major molecular response (MMR)] *(7)*. Further reductions in MR from a standardized baseline [the "interna-

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[9] Nonstandard abbreviations: TKI, tyrosine kinase inhibitor; CML, chronic myeloid leukemia; RT-qPCR, reverse transcription–quantitative PCR; %*BCR-ABL1*^{IS}, percentage *BCR-ABL1*^{IS}; MMR, major molecular response; IS, international scale; cDNA, complimentary DNA; dPCR, digital PCR; MIQE, minimum information for publication of quantitative real-time PCR experiments; dMIQE, digital MIQE; WMRGL, West Midlands Regional Genetics Laboratory; SNB, Sample Negative Birmingham; SNH, Sample Negative Hammersmith; LoD, limit of detection; QS3D, QuantStudio™ 3D Digital PCR System; FPR, false positivity rate.

[10] Human genes: *BCR-ABL1,* BCR, RhoGEF and GTPase activating protein–ABL proto-oncogene 1, non-receptor tyrosine kinase; *ABL1,* ABL proto-oncogene 1, non-receptor tyrosine kinase; *GUSB,* glucuronidase beta.

tional scale" (IS)] are defined as MR^4 [detectable disease ≤0.01% *BCR-ABL1*^{IS} or undetectable disease in complimentary DNA (cDNA) with ≥10 000 *ABL1* transcripts], and $MR^{4.5}$ (detectable disease ≤0.0032% *BCR-ABL1*^{IS} or undetectable disease in cDNA with ≥32 000 *ABL1* transcripts) *(7)*.

While expressing the *BCR-ABL1* transcript levels as a percentage of the total number of *ABL* transcripts on the IS has improved interlaboratory comparisons *(8–9)*, it requires regular and cumbersome standardization. Digital PCR (dPCR) is an adaption of the qPCR method that could enable simple, standardized quantification of nucleic acids, primarily due to its nonreliance on calibration curves *(10)*, that could improve accuracy when measuring RNA transcripts *(11)*. dPCR was used to quantify the ERM®-AD623 reference material that can be used either for the calibration of secondary "inhouse" control materials or to traceably calibrate *BCR-ABL1* copy numbers *(12)*. dPCR can also be used directly for the absolute quantification of the *BCR-ABL1* copy numbers. In this study, we compared 3 different dPCR platforms and investigated whether they could be applied in a clinical setting to quantify *BCR-ABL1* transcripts in CML patients.

Materials and Methods

All reverse transcription, qPCR and dPCR experiments were in accordance with the minimum information for publication of quantitative real-time PCR experiments (MIQE) and digital MIQE (dMIQE) guidelines (see Table S1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol63/issue2) *(13–14)*.

MATERIALS

The ERM-AD623 (Sigma-Aldrich) plasmid produced by the European Commission for Reference Materials *(12)* in addition to an inhouse control, the "Wessex" plasmid (provided by the National Genetics Regional Laboratory), were used for the performance evaluation of RT-dPCR. Both plasmids contain identical *BCR-ABL1*, *ABL1*, and glucuronidase beta (*GUSB*) sequences. Aliquots of each were stored at −20 °C. Fresh dilutions of between 50 000 copies/ μL and 2 copies/μL were prepared from the ERM plasmid, while dilutions of between 40 000 copies/3 μL and 10 copies/3 μL were prepared from the Wessex plasmid. The latter was routinely used for the RT-qPCR calibration curve.

CLINICAL SAMPLES

In total 102 clinical samples were analyzed in this study. Ethical approval was provided and informed consent was obtained from all patients in accordance with the Declaration of Helsinki. Archived cDNA material from 70 CML clinical samples encompassing 7 different disease levels (as measured by RT-qPCR and expressed as a percentage of *BCR-ABL1*^{IS}) were used: 20%, 10%, 1%, 0.1%, 0.01%, 0.001%, and <0.001% *BCR-ABL1* (see online Supplemental Table S2). A further 32 non-CML samples were used as negative controls. These were obtained from patients referred to the West Midlands Regional Genetics Laboratory (WMRGL) [Sample Negative Birmingham (SNB): 19 samples] and Hammersmith Hospital, London [Sample Negative Hammersmith (SNH): 13 samples] for investigation of myeloproliferative neoplasms; they were classified as non-CML based on cytogenetic and PCR tests (see online Supplemental Table S2). Negative controls from healthy volunteers were not included for practical reasons and because we felt these would add no further information to the investigation of the assay's limit of detection (LoD).

All cDNA samples were stored at −80 °C at the Hammersmith Hospital (for a median of 2 years, range 1–7 years) following the processing of peripheral blood samples as they arrived at the laboratory according to a previously described protocol *(15–16)*. Briefly, 20–30 mL of peripheral blood was washed in red cell lysis buffer and the total white blood cell pellet was lysed in 1.5 mL RLT buffer (Qiagen) and stored at −80 °C. RNA was extracted from 350 μL of the RLT lysate using the Qiagen RNeasy Mini kit (Cat No. 74106, Qiagen) following the manufacturer's protocol on the QIAcube robotic workstation (Qiagen). Reverse transcription was performed using 480U of M-MLV reverse transcriptase (Invitrogen, Cat No. 28025013) and 0.6 μg of random hexamers (Invitrogen, Cat No. N8080127) with 55 μL of RNA added in a 100 μL final reaction. To maintain routine feasibility, the RNA was added based on volume, with no correction for RNA concentration, and was neither evaluated for RNA quality nor DNase treated, as is normal practice in our diagnostic laboratory. Nevertheless, as part of the method development, RNA from a subset of samples was reextracted from the RLT lysates using the Qiagen RNeasy Mini plus kit (Qiagen) including an on-column DNase digestion step.

BCR-ABL1 AND *ABL1* ASSAYS

Both the *BCR-ABL1* and *ABL1* assays used in this study were published by the EAC (Europe Against Cancer) initiative *(17–18)*. For the RT-qPCR, QuantStudio™ 3D Digital PCR System (QS3D) and QX200 platforms, both assays were modified to allow a minor groove binding (MGB) quencher to be conjugated to the 3′ end of the hydrolysis probes as described previously *(19)* (see online Supplemental Table S3). For the RainDrop platform, a TET (tetrachlorofluorescein) quencher was conjugated to the 3′ end of the hydrolysis probe (see online Supplemental Table S3). Assay optimization was per-

formed with primer and/or probe titrations and annealing temperature gradients for the 3 RT-dPCR platforms.

QUANTSTUDIO™ 3D dPCR SYSTEM

QS3D experiments were performed at Hammersmith Hospital. For each sample, 15 μL reactions were prepared containing 1× QS3D Digital PCR Master Mix, 900 nmol/L each of forward and reverse primer and 250 nmol/L of each of the probes. For analysis of both the reference material and clinical samples, 1 μL of cDNA was added to each reaction. A 2-fold dilution series (512–2 copies per reaction) and a 5000-increment dilution series (50000–1000 copies per reaction) were prepared from the reference material. In the clinical samples, triplicate RT-dPCR reactions were performed for the 4 highest disease levels (20%, 10%, 1%, and 0.1%) and 9 replicate reactions were performed for the lower disease groups and controls. For NTCs, 1 μL of nuclease-free water was added in place of cDNA.

The reaction mix was loaded into a QS3D Digital 20K chip (V1) according to the manufacturer's instructions and placed in an ABI 9700 thermocycler (Thermo Fisher Scientific). Thermocycling conditions were 96 °C for 10 min, followed by 40 cycles at 55 °C for 1 min and 98 °C for 30 s and a final extension at 55 °C for 2 min with a 16 °C hold. The chips were read using the QS3D reader and analyzed using the online Analysis Suite™ v1.0. Using the 2D scatter plots and histograms in control reactions, thresholds were set in both channels (FAM and VIC) to classify the partitions containing positive or negative amplification for the 2 targets.

QX200™ DROPLET dPCR SYSTEM

QX200 experiments were performed at LGC, London. For each sample, 20 μL reactions were prepared containing 1× Bio-Rad RT-dPCR SuperMix, 900 nmol/L each of forward and reverse primer, 250 nmol/L of each probe in a duplex reaction and 1 μL of cDNA. As with the QS3D, triplicate reactions were performed for samples in the 4 highest disease level categories and 9 replicate reactions were performed for the lower disease groups and controls. For NTCs, 1 μL of nuclease-free water was added in place of cDNA. Droplets were generated according to the manufacturer's instructions. Thermocycling was performed with a C1000 Touch thermocycler (Bio-Rad). Thermocycling conditions were 96 °C for 10 min, followed by 40 cycles at 94 °C for 30 s and 60 °C for 1 min. A final incubation at 98 °C for 10 min was performed for signal stabilization followed by a 12 °C hold. The ramp rate was 2 °C/s. Droplets were counted and fluorescence determined using the QX200 Droplet Reader and data was analyzed using the QuantaSoft v.7.0.1 software. Using the 2D scatter plots and control reactions, thresholds were set in both channels to classify the positivity for the 2 targets.

RAINDROP DROPLET dPCR SYSTEM

RainDrop experiments were performed at the WMRGL. For each sample, 40 μL reactions were prepared containing 1× Applied Biosystems® TaqMan® Universal PCR Master Mix, 900 nmol/L of each primer, 200 nmol/L of each probe, 1× Droplet Stabilizer, and 10 μL of cDNA. Each sample was measured using duplicate reactions regardless of the disease level. For NTCs, 10 μL of nuclease-free water was added in place of cDNA. Droplets were generated using the RainDrop Source according to the manufacturer's instructions and collected in an Axygen 8 well tube strip (PN PCR-0208-C). Thermocycling was performed with a C1000 Touch thermocycler (Bio-Rad). Thermocycling conditions were 95 °C for 10 min, followed by 49 cycles at 95 °C for 15 s and 60 °C for 1 min. A final incubation at 98 °C for 10 min was performed followed by a 10 °C hold. Droplets were counted using the RainDrop Sense. Data were analyzed using the RainDrop Analyst software.

Different cDNA volumes were used on different dPCR platforms because the platforms have different reaction volumes to which different amounts of cDNA can be added with the larger reaction volume and partition number of the Raindrop platform allowing more template to be added. One microliter of cDNA on the dPCR platforms with 20 000 partitions represented a pragmatic volume that enabled an optimal concentration of *ABL1* to be measured without saturating the reactions.

QUANTITATIVE RT-qPCR

RT-qPCR experiments were performed at Hammersmith Hospital using the 7900 ABI qPCR system as described previously *(19)*.

ABSOLUTE COPY NUMBER MEASUREMENT

Files were exported from each RT-dPCR experiment containing the number of positive partitions (k) for each assay and the total number of partitions (n) per reaction. These numbers were used to estimate the number of copies per partition (λ) using the equation $\lambda = -\ln(1 - k/n)$ *(13, 20–21)*. The *BCR-ALB1* and *ABL* concentration in the reference materials and cDNA was calculated by converting λ to copies/μL:

$$\text{Copies per } \mu\text{L} = \frac{\lambda}{V_p} = \frac{\text{Total reaction volume}}{\text{Added template volume}}$$

Where V_p is the partition volume (μl) as published by the manufacturer (QS3D; 0.000809 μL, QX200; 0.00085 μL, and RainDrop; 0.000005 μL). The percentage of *BCR-ABL1* was calculated as the ratio between the number of copies of *BCR-ABL1* and *ABL1*. Estimated target copy numbers per reaction were calculated by multiplying the copies/ μL by the total reaction volume.

STATISTICAL ANALYSIS

Graphing and basic statistical analysis including calculation of means, variances, SDs, CIs, and *P* values were performed using Microsoft Excel 2013, GraphPad Prism 6.0, and the R statistical programming environment (http://www.r-project.org/). For all statistical analyses, $P < 0.05$ was considered significant. The workflow of the statistical analysis is shown in (see online Supplemental Fig. S1).

Results

PERFORMANCE EVALUATION OF THE DUPLEX *BCR-ABL1* AND *ABL1* ASSAY BY RT-dPCR

Before the clinical analysis, validation experiments were performed using the QS3D platform. The aim was to assess the performance characteristics of a clinically applied RT-qPCR assay on an RT-dPCR platform. Dilutions of the ERM-AD623 and Wessex plasmids were used to measure and compare the 2 targets (*BCR-ABL1* and *ABL1*). Performance characteristics evaluated included the reaction set up (primer-probe concentrations, annealing temperatures, and comparison of uniplex and duplex reactions; also assessed on the other 2 digital platforms), linearity and sensitivity of the assay, and identification of bias between the 2 targets (see online Supplemental Figs. S2–S6).

Using the ERM-AD623 reference material, duplex reactions were performed with comparable results to uniplex reactions. There was no significant difference in measured concentrations ($P = 0.385$) (see online Supplemental Fig. S6A). For the investigated range, the ratio between the 2 targets showed no significant deviation from the expected ratio of 1 ($P = 0.471$; see online Supplemental Fig. S6B). Therefore, duplex experiments were used for the remainder of the study.

Linearity was maintained across the dilution range for both targets with an R^2 of 0.99 (see online Supplemental Fig. S6C). A dilution of the inhouse Wessex plasmid was set up and measurements compared to those obtained with the ERM-AD623 standard. As the concentration of the former was determined by spectrophotometry, the comparison with the ERM-AD623 reference material was used to determine if a conversion factor was needed. A highly significant systematic bias of approximately 50% was identified with the Wessex plasmid compared with the ERM-AD623 ($P < 0.0001$) (see online Supplemental Fig. S7A). A similar bias was also identified in the ERM evaluation study *(12)* and explained by the assignment of copy numbers assuming a single-stranded Wessex plasmid, but double stranded ERM plasmid. Using Bland–Altman Ratio plots a conversion factor of 0.46 was calculated and used for the remainder of the study. Importantly, there was no significant difference in the ratio of both targets (see online Supplemental Fig. S7B).

EVALUATION OF dPCR FOR QUANTIFICATION OF CLINICAL SAMPLES

For all clinical samples *BCR-ABL1* and *ABL1* transcripts were measured by RT-qPCR and RT-dPCR on same cDNA samples, and the percentage ratio between the 2 targets was compared. For the QS3D platform, 2 negative *ABL1* populations were observed in all samples. These were not clearly separated from each other, but obviously distinct from the positive population of partitions (see online Supplemental Fig. S8). An investigation of a subset of cDNA samples treated with and without DNase, in addition to using different *ABL1* assays, identified the cause of this problem to be genomic DNA contamination from the extraction process. Therefore, in all QS3D analyses, the additional negative populations were included in the negative partition count by adjusting the value of the fluorescent intensity threshold (see online Supplemental Fig. S8). This was not observed in experiments with the 2 droplet platforms.

ABL1 transcript copy numbers measured all 3 RT-dPCR platforms showed good correlation with RT-qPCR across all sample groups ($R^2 = 0.91$, 0.93, and 0.95 for QS3D, QX200, RainDrop, respectively; Fig. 1A). RT-dPCR quantification of the *BCR-ABL1* transcript copy numbers correlated well with RT-qPCR across all 3 platforms only down to ≥0.1% ($R^2 = 0.85$, 0.94, and 0.92 for QS3D, QX200, RainDrop, respectively; Fig. 1B). Below 0.1%, while the copy number correlations between RT-qPCR and the 3 RT-dPCR measurements were maintained, RT-dPCR produced a greater variation in the replicate measurements for each sample which was not observed for the RT-qPCR counterpart measurements. Interestingly, copy number concentrations for *BCR-ABL1* transcripts were similar across the 4 platforms whilst *ABL1* concentrations showed more variability. As $\%BCR\text{-}ABL1^{IS}$ is intrinsically linked to copy number concentrations for both targets, the increase in variability of the *ABL1* transcripts resulted in the $\%BCR\text{-}ABL^{IS}$ frequently varying by 1 order of magnitude when compared to RT-qPCR ($R^2 = 0.89$, 0.92, 0.97 for QS3D, QX200, RainDrop, respectively; Fig. 1C). Furthermore, none of the platforms were able to substantially improve the sensitivity of quantification by RT-qPCR in patient samples with $\%BCR\text{-}ABL1^{IS}$ level <0.001%.

When using the QS3D there was good correlation down to approximately 20 copies of *BCR-ABL1* transcripts, below which a plateau was observed (Fig. 2). For quantification of the lowest 2 residual CML categories, 0.001% (where RT-qPCR returned a consistent value of 1 copy per 3 μL cDNA for all 10 patients) and <0.001% (where the qPCR results were negative), all 3 RT-dPCR

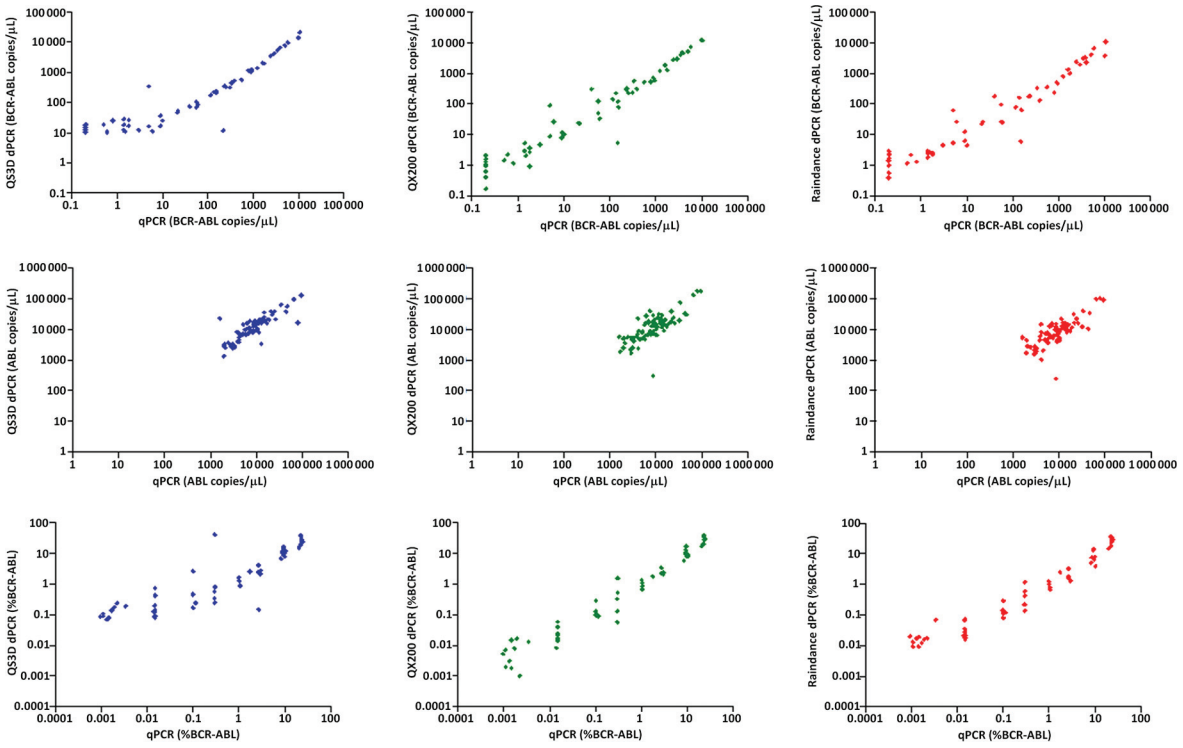

Fig. 1. Comparison of RT-qPCR with RT-dPCR for the quantification of *BCR-ABL1* and *ABL1* transcript copy numbers in clinical samples.

Scatter plots demonstrating the linear relationship between the quantification of (A), *BCR-ABL1*, (B), *ABL1*, and (C), %*BCR-ABL1*^{IS}. Quantification of the cDNA derived from clinical samples by RT-qPCR (*x*-axis) was compared with the QS3D (blue), QX200 (green), and RainDrop (red) platforms. Each data point represents the mean value derived from either triplicate or 9 replicate reactions. Correlation coeffiencies were as follows: (R^2 = 0.91, 0.93, 0.95 for QS3D, QX200, RainDrop, respectively) for *ABL1* transcript copy numbers; (R^2 = 0.85, 0.94, 0.92 for QS3D, QX200, RainDrop, respectively) for *BCR-ABL1* transcript copy numbers; (R^2 = 0.89, 0.92, 0.97 for QS3D, QX200, RainDrop, respectively) for %*BCR-ABL1*^{IS}.

instruments detected amplification of *BCR-ABL1* targets (Fig. 2). However, the non-CML control groups also showed a positive measurement for *BCR-ABL1* that rendered the measurements obtained in the low disease groups indistinguishable from normal background noise (Fig. 2). As expected, RT-qPCR was consistently negative with no *BCR-ABL1* transcripts measured in the <0.001% category (samples in this category were chosen based on a zero reading by RT-qPCR), but detected 1–3 copies in some of the control patient groups and NTCs. The QS3D and Raindrop platforms had an appreciable false positivity rate (FPR) in the NTC reactions while only a single positive droplet was observed in 1 of the 30 NTC reactions using the QX200 platform (Fig. 2).

For the QX200 platform, the plateau was less pronounced than that observed with the QS3D. The FPR was visibly lower than those of the QS3D and Raindrop platforms. However, for the QX200, the readings obtained from the lowest 3 CML categories (≤0.01%) were indistinguishable from the negative control patient groups (Fig. 2). Only the Raindrop platform was able to measure % *BCR-ABL1* ^{IS} in the <0.001% range at a level higher than the FPR in the control patient groups (Fig. 2). This was possibly related to the larger volume of cDNA added to each reaction (10 µL as opposed to 1 µL). However, in the CML patient samples, the Raindrop platform did not identify any difference in the *BCR-ABL1* transcripts compared to the disease level they were assigned by RT-qPCR (0.01%, 0.001% or <0.001%; Fig. 2).

We explored the effect of differing quantities of input cDNA on sensitivity by performing 9 replicate reactions on the dPCR platforms with 20 000 partitions (i.e., using a total of 9 microliters of cDNA) for samples known to be below MMR (0.1% IS) as a mean for testing a larger volume of cDNA per sample. This increased the quantitative precision but not the sensitivity of the tests.

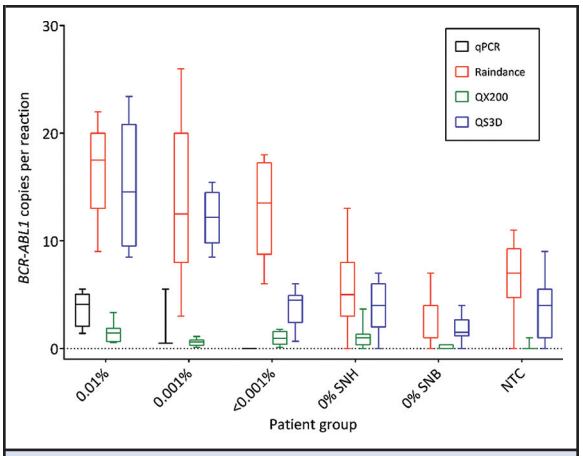

Fig. 2. Comparison of the *BCR-ABL1* transcript copy numbers measured per reaction in low disease level patient (≤0.01%) and matched control samples.

The 3 low disease level patients as measured by RT-qPCR (0.01%, 0.001%, and <0.001%) are shown with the matched non-CML controls (SNH and SNB) that are nominally 0% *BCR-ABL1*^{IS}. The NTC reactions were generated with water in place of cDNA. The box plots show the median and interquartile range with the whiskers showing the minimum and maximum data points from 9 replicates for each platform. The dashed horizontal line indicates zero *BCR-ABL1* transcript copies per reaction.

Discussion

In this study, we assessed the performance of 3 different RT-dPCR platforms for the future application of measuring %*BCR-ABL1*^{IS} levels in response to TKI therapy and compared each with the RT-qPCR. RT-dPCR can perform absolute quantification by counting cDNA molecules and has been reported to offer high precision and accuracy *(22–25)* as well as being described as "calibration-free" *(26)*. While these strengths of RT-dPCR have facilitated its use in assigning specific values to reference materials *(12, 27–28)*, we wanted to explore whether this method had the potential to be applied clinically to CML samples. For this purpose, we compared the gold-standard RT-qPCR test to 3 RT-dPCR instruments. For patients with a relatively high level of disease (<0.1% *BCR-ABL1*) the measurements provided by the different platforms were highly concordant. However, we identified false positivity in all 3 RT-dPCR platforms, particularly at levels <0.01%, that precluded their use as a more sensitive alternative to RT-qPCR.

Taken together the data presented here suggest that RT-dPCR did not appreciably improve the sensitivity of % *BCR-ABL1*^{IS} measurement when an equal amount of cDNA was investigated on both platform types. However, the main advantage of RT-dPCR over RT-qPCR is that it can be performed without the requirement for a calibration curve, thus offering a simpler means of ensuring interlaboratory reproducibility. In addition, RT-dPCR may offer greater confidence in the detection of low *BCR-ABL1* copy number concentrations at the limits of the current RT-qPCR technology; this could allow better distinction of patients in MR^4 (<0.001% *BCR-ABL1*^{IS}) and MR$^{4.5}$ (<0.0001% *BCR-ABL1*^{IS}) in patients being considered for treatment cessation trials. The expected improvement in performance and reduction in reagent costs over the next 3–5 years is therefore likely to contribute to the implementation of dPCR on a larger scale.

Normalization using the *ABL1* gene appeared to confound the results as there was generally a better agreement between RT-dPCR and RT-qPCR when measuring *BCR-ABL1* absolute values than for *ABL1* (Fig. 1). This would suggest that while *ABL1* may be a good choice of a reference gene for RT-qPCR *(18)*, selection of an alternative reference gene following MIQE and dMIQE guidelines might be prudent if RT-dPCR is to be adopted into clinical diagnostics *(13–14)*.

The findings of our study suggest that RT-qPCR is already working close to the physical LoD. To improve sensitivity using RT-dPCR, an increase in the amount of template would be required; this could be achieved either by the addition of more cDNA to the reaction, or by increasing the reaction volume. Other changes of potential benefit include the reduction of background noise to reduce the false positivity in the negative control sets. Further work is required to elucidate the causes of false positivity. The reproducible identification of both target molecules in the negative samples from Hammersmith hospital on both platform types after the reextraction of RNA from the original GTC lysates, confirmed that the false positivity was not caused by contamination of the original cDNA material. Certainly, RT-dPCR is at a greater risk of false-positive findings compared with RT-qPCR as the large numbers of partitions analyzed for each reaction increases the chance that a small number of these partitions will result in high fluorescence after 40 cycles of amplification. A concerted effort by the molecular testing community will be required to clarify the cause of these events.

Conclusion

The main findings of this study illustrate that RT-dPCR has a comparable performance to RT-qPCR over a 4 log dynamic range for the quantification of *BCR-ABL1*. To further improve assay sensitivity and measure MR^4 and MR$^{4.5}$ with confidence, changes to the upstream processing are required. The unique format of RT-dPCR, which essentially counts cDNA molecules, allows us to conclude that a major reason for this is that RT-qPCR is already measuring close to the single molecule level. Protocol modifications would therefore be required to phys-

ically increase the number of cDNA molecules made available to the PCR to increase this limit. RT-dPCR offers a powerful method to evaluate these modifications to improve RT-qPCR in the short term, however with further optimization and correct selection of reference gene, RT-dPCR could provide a reproducible alternative method to RT-qPCR in future.

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