Monitoring BCR-ABL1 fusion transcripts by real-time quantitative RT-PCR has become an important clinical test for the management of patients with chronic myeloid leukemia. However, it has some inherent limitations with regard to its lower limit of detection and limit of quantification. Improvement in the lower limit of detection could aid clinicians in selecting candidates for discontinuation of tyrosine kinase inhibitors without relapse. Improvement in the limit of quantification may also avoid unnecessary testing or changes in therapy. Here, we demonstrate the advantages of droplet digital RT-PCR with regard to simplicity, lower limit of detection, and limit of quantification. We expect the advantages of droplet digital RT-PCR will make it the preferred method for quantification of BCR-ABL1 fusion transcripts. (J Mol Diagn 2014, 16: 174–179; http://dx.doi.org/10.1016/j.jmoldx.2013.10.007)

Several studies have demonstrated the clinical utility of quantifying BCR-ABL1 fusion transcript levels in chronic myeloid leukemia (CML) patients. Typically, this has been performed using real-time quantitative RT-PCR (RT-qPCR). However, as a result of the rapid adoption of BCR-ABL1 transcript monitoring by clinical laboratories, testing methodologies vary considerably, as does the analytical performance. Efforts to harmonize methods and reporting have been underway for several years and have been aided by the manufacturing of commercially available calibrators. Nevertheless, analytical performance continues to vary considerably across laboratories because much of this variability may be inherent in the RT-qPCR methodology.

The lower limit of detection (LOD) of the RT-qPCR method may be insufficient for managing CML patients who have had a good response to tyrosine kinase inhibitors (TKIs). The International Randomized Study of Interferon and STI571 (IRIS) trial defined a major molecular response as a 3-log reduction in BCR-ABL1 transcript levels from a median value at diagnosis, and this 3-log reduction has been defined as 0.1% on the International Scale (IS). Subsequently, a complete molecular response has been defined by the European LeukemiaNet as undetectable BCR-ABL1 transcripts in two consecutive, high-quality samples by a test with an LOD of 0.01% IS. However, an even lower LOD may be necessary to manage those patients who may be eligible for TKI cessation. Several investigators have explored the discontinuation of imatinib for patients who had maintained a complete molecular response for an extended period of time. In all cases, regardless of the center monitoring BCR-ABL1 fusion transcripts, some patients maintain long-term remission, whereas many others relapse, indicating that the LOD for these tests is insufficient to differentiate these two groups of patients. Given the greater therapeutic responses exhibited with second-generation tyrosine kinase inhibitors, a larger proportion of patients can be expected to reach complete molecular response, and therefore, a more exact definition of complete molecular response and a lower LOD will be necessary to select candidates for discontinuation of TKIs with lower risk of relapse.

The accuracy of current RT-qPCR methods, especially at the lower limits of quantification (LOQ), may also affect the clinical decisions on how CML patients are monitored and therapeutically managed. Rising BCR-ABL1 fusion transcript levels require more frequent patient follow-up and testing to detect development of therapy resistance and to exclude noncompliance to TKI therapy. How high the
fusion transcript level must rise before it is considered biologically (and therefore clinically) relevant is not clear. Published recommendations range from an increase of 0.3 to 0.5 log (ie, a 200% to 316% increase).10,25–27 These recommendations, unfortunately, are based on the degree of accuracy of the RT-qPCR method and not on biological variation. Therefore, improvement in the accuracy of quantification, especially at the lower levels of detection and therapeutic thresholds (eg, major molecular response) could greatly affect the management of these patients by allowing closer monitoring of those with biologically relevant changes in fusion transcript level, and avoiding unnecessary follow-up and monitoring in those without.

The advantages of digital PCR for quantifying DNA copy number and rare events have been highlighted previously.28–35 As compared to quantitative analog PCR, digital PCR gives the absolute copy number, so standard curves are not necessary and data are more accurate. Therefore, we developed a droplet digital RT-PCR (RT-ddPCR) test to quantify BCR-ABL1 fusion transcripts to demonstrate its advantages over the widely adopted conventional RT-qPCR.

Materials and Methods

Samples

With approval of the institutional review board, previously extracted total RNA from pre- and post-treatment clinical samples were de-identified and pooled to create a single sample. Specifically, a diagnostic sample with a relative BCR-ABL1 transcript ratio near the laboratory median was pooled with nine follow-up samples that had undetectable BCR-ABL1 transcript levels (LOD >4 log). This pooled sample was tested and compared to ARQ IS calibrator panels (Asuragen, Austin, TX) to show an undiluted concentration of 10% on the IS. The diluent RNA was obtained from a healthy volunteer, and total RNA was extracted using the QIAamp RNA Blood Kit by Qiagen (Cat#52304; Qiagen, Valencia, CA). The RNA concentration and purity was assessed using a NanoDrop spectrophotometer by NanoDrop Technologies (Wilmington, DE). Each of the ARQ IS calibrator panels (e13a2 and e14a2) consists of four diluted calibrators at 10%, 1%, 0.1%, 0.01%, and a negative control.

Preparation of Dilutions

To assay for the BCR-ABL1 fusion transcript, the pooled RNA was serially diluted into the diluent RNA. Using 100 ng/μL for the pooled RNA and diluent, a 10-fold dilution series was performed to make the following concentrations: 10^0, 10^-1, 10^-2, 10^-3, 10^-4, and 10^-5. Given the targeted starting concentration of 10% IS, these correspond to the following percentage IS values: 10%, 1%, 0.1%, 0.01%, 0.001%, and 0.0001%, respectively. The diluent RNA also served as a negative control. To assay for BCR, a similar dilution was performed, but the pooled and diluent RNA concentration was 8 ng/μL. The ARQ e13a2 and the ARQ e14a2 panels consisted of four diluted calibrators at 10%, 1%, 0.1%, 0.01%, and a negative control. To assess the lower LOD, a 0.001% IS dilution was generated by making a 1:10 dilution of 0.01% IS with the negative RNA that was provided with each ARQ IS calibrator panel.

Design of Primers and Probes

TaqMan MGB probes and primers (Life Technologies, Carlsbad, CA) were generated for the BCR-ABL1 fusion transcripts (e13a2 and e14a2) and the BCR transcripts (Table 1). Human mRNA sequences for BCR-ABL1 (e13a2 and BCR and BCR transcripts in the ARQ IS calibrator kits. The sequence of interest was transferred to Primer Express version 2.0 software (Applied Biosystems, Life Technologies) for probe and primer generation. Within Primer Express, the setting of the TaqMan MGB probe and primer design was used to generate probes and primers. The BCR-ABL1 probe was generated with a FAM reporter, and the BCR probe was generated with a VIC reporter.

Droplet Digital PCR

Before the RT-PCR reaction mixture assembly, all of the RNA samples, including the ARQ IS calibrator panel, were incubated for 5 minutes at 75°C. The BCR-ABL1 and BCR transcripts were tested separately in different wells to account for the difference in input total RNA. The RT-PCR reaction mixture was loaded into the Bio-Rad Laboratories (Cat# 1863021; Hercules, CA). The BCR primer and probes were at final concentrations of 900 and 250 nmol/L, and 40 ng of template in a final volume of 20 μL. The BCR-ABL1 primer and probes were at final concentrations of 2250 and 625 nmol/L, and 600 ng of template in a final volume of 20 μL. The 20-μL droplet digital PCR (ddPCR) reaction mixture was then loaded into the Bio-Rad DG8 disposable droplet generator cartridge. A volume of

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
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<tbody>
<tr>
<td>BCR-ABL1 forward</td>
<td>5’-CATTCGCTGACCATCAATA-3’</td>
</tr>
<tr>
<td>BCR-ABL1 reverse</td>
<td>5’-ACACCATCCCACTTTGAT-3’</td>
</tr>
<tr>
<td>BCR-ABL1 probe</td>
<td>ATCTGA-3’</td>
</tr>
<tr>
<td>BCR forward</td>
<td>5’-CAGTGGAGGAGAGATCGA-3’</td>
</tr>
<tr>
<td>BCR reverse</td>
<td>5’-CATGGCTCCTCCTCCGAA-3’</td>
</tr>
<tr>
<td>BCR probe</td>
<td>5’-VIC-CAGCGCTCAGCTCAA-3’</td>
</tr>
</tbody>
</table>
70 µL of droplet generation oil was loaded into the oil well for each sample. The cartridge was placed into the droplet generator. The generated droplets were transferred to an Eppendorf 96-well twin.tec PCR plate (Eppendorf, Hamburg, Germany). The plate was heat-sealed with a Thermo Scientific Easy Pierce pierceable foil seal from Thermo Fisher Scientific (Cat#AB-0757; Waltham, MA) and then placed on a thermal cycler and amplified to the endpoint. Thermal-cycling conditions were 60°C × 30 minutes (1 cycle), 95°C × 5 minutes (1 cycle), 95°C × 30 seconds (ramp rate 2.5°C/second), and 59°C × 60 seconds (ramp rate 2.5°C/second) (40 cycles), 98°C × 10 minutes (1 cycle), and a 12°C hold.

After PCR, the 96-well PCR plate was loaded on Bio-Rad’s QX100 droplet reader, which reads the droplets from each well of the plate. Analysis of the ddPCR data was performed with QuantaSoft analysis software version 1.3.2.0 that accompanied the QX100 droplet reader.

This system partitions the sample into an emulsion of up to 20,000 stable, nanoliter droplets. The emulsion is thermocycled, and each droplet serves as an independent reactor for PCR. After PCR, each droplet is read with a two-color fluorescence detector to determine how many droplets have positivity for the BCR-ABL1 fusion transcript, BCR transcript, or both. For these studies, BCR-ABL1 and BCR were analyzed separately so that more RNA could be added to the BCR-ABL1 wells and rare events could be detected. Replicates were tested by two technologists over 20 days.

**Results**

Analytical performance was assessed using IS calibrators and dilutions of the pooled clinical samples. A total of six replicates of each concentration were performed except for the lowest concentration of the IS calibrator (0.001%), which was tested 9 times, and the negative sample, which was tested 21 times. Samples were blinded, and replicate measures were performed by two technologists over 20 days. Data from both the calibrators and dilutions showed remarkable linearity, trueness, and precision down to 0.001% (Figures 1 and 2, and Tables 2 and 3). After converting to log-log scale, linear regression showed no concentration-dependent bias, and R² equaled 0.9995 and 0.999 for the calibrators and dilutions, respectively. Moreover, the dilutions of the pooled diagnostic sample demonstrated near perfect correlation to the calibrators. All negative samples were entirely negative.

Measurements of the lowest concentrations (0.001% IS calibrator and 10⁻⁶ dilution) had some replicates that were positive and others that were negative, indicating that these concentrations were beyond the LOD for a single well. Because the negative samples showed no background, even a single positive droplet per well can be detected. Therefore, by combining the counts from multiple replicate wells, the limits of detection and quantification can be extended further, and the copies of BCR-ABL1 transcripts in a larger sample volume can be quantified. For example, as demonstrated with the dilution samples, by combining the counts of six wells, the LOD can be lowered to 0.0001% IS although the quantification is inaccurate. As demonstrated with the IS calibrators, by combining the counts of nine
wells, the LOQ of the calibrators can be lowered to 0.001%. The differences in the LOD for these sample types reflect the differences in amplifiable RNA that is added to each well. For this assay, we determined that 600 ng of total RNA can be added to a single well without affecting the accuracy (data not shown), so for the dilution samples, 600 ng was added to each well. For the IS calibrator samples, 5 µL was added to each well. For comparison, the number of copies of BCR in the dilution samples was approximately 366 copies/40 ng (or approximately 5489 copies/600 ng), whereas the number of copies of BCR in the IS calibrators was approximately 2750 copies/5 µL. Therefore, 1 µL of the IS calibrator is equivalent to approximately 60 ng of total RNA. If a lower LOD or more accurate data were required, the counts from additional wells could be combined, provided sufficient amplifiable RNA were available.

### Discussion

Herein, we demonstrate the advantages of RT-ddPCR for quantifying BCR-ABL1 fusion transcripts as compared to the widely adopted standard method, RT-qPCR. The most important advantages are improvement in simplicity, LOD, and LOQ.

The advantages of digital PCR were first described 20 years ago. At that time, limiting dilutions, PCR, and Poisson statistics were used to quantitate the total number of amplifiable targets within a sample. The recent introduction of ddPCR has greatly simplified the process. With ddPCR, samples are partitioned into nanoliter droplets so that each droplet becomes a separate sample with zero, one, or more copies of the target DNA molecule. The droplets are thermally cycled to endpoint, and each droplet is read to determine the fraction of positive droplets. Using Poisson statistics, the absolute number of starting copies can very accurately be determined. Because ddPCR uses endpoint detection of the amplification product, the efficiency of amplification is much less of a concern, and calibration curves are not necessary. The advantages of digital PCR with regard to test development, LOD, and accuracy were highlighted previously.

Using IS calibrators and dilutions of pooled clinical samples, we demonstrate an LOD of 0.001% and 0.0001%, respectively. The difference in the LOD for these sample types is a reflection of the total amount of RNA tested. For the IS calibrators, 5 µL of sample was added per well (equivalent to approximately 300 ng of total RNA), and for the dilution samples, 600 ng of sample was added per well. Given the negative background, higher concentrations per well and/or more wells per sample would theoretically allow one to extend the LOD even lower. Well-optimized RT-qPCR assays cannot achieve the LOD demonstrated here. For RT-qPCR, interference from nontarget RNA and nonspecific amplification prevents detection of very rare transcripts. For example, a well-optimized assay may be able to detect 10 copies per well.\(^\text{14}\) However, RT-ddPCR partitions the sample into nanoliter droplets, thus minimizing interference from nontarget RNA and nonspecific amplification so that even a single, rare event can be detected. Therefore, with a negative background, the LOD using RT-ddPCR is only limited by the amount of patient material that is tested.

Perhaps the most important advantage of RT-ddPCR over RT-qPCR is the improvement in quantitation. Here, we demonstrate absolute copy number determination without bias as compared to the IS calibrators. Furthermore, for those samples with concentrations greater than the LOD for a single well, the coefficient of variation ranged from 8% to 23% for the clinical dilution series, and 15% to 30% for the IS calibrators. By combining wells, the degree of imprecision can be reduced even further. This level of precision is far better than that achieved with well-optimized RT-qPCR assays.\(^\text{8,38-40}\) In a multi-institutional study to harmonize BCR-ABL1 quantification by RT-qPCR and reporting on the IS, only 58% of centers achieved a bias within 1.2-fold and an imprecision less than ±5-fold.\(^\text{10}\)

The use of digital PCR to detect BCR-ABL1 fusion transcripts has been reported previously.\(^\text{15}\) For that study, digital PCR was used in conjunction with a pre-amplification step. Digital PCR was performed using the BioMark Real-Time PCR System (Fluidigm, South San Francisco, CA), which partitions the sample into 765 separate wells. The investigators were able to demonstrate a 2- to 3-log improvement in the LOD as compared to conventional RT-qPCR.

### Table 2: Analytical Performance Parameters of RT-ddPCR

<table>
<thead>
<tr>
<th>(N)</th>
<th>Target value (%)</th>
<th>Data mean</th>
<th>SD</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>10</td>
<td>0.20482</td>
<td>0.03111485</td>
<td>15</td>
</tr>
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<td>6</td>
<td>1</td>
<td>0.0239796</td>
<td>0.0039164</td>
<td>16</td>
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<tr>
<td>0.1</td>
<td></td>
<td>0.0023995</td>
<td>0.0006142</td>
<td>26</td>
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<tr>
<td>6</td>
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<td>0.0002233</td>
<td>6.693 × 10⁻⁵</td>
<td>30</td>
</tr>
<tr>
<td>9</td>
<td>0.001</td>
<td>2.023 × 10⁻⁵</td>
<td>2.928 × 10⁻⁵</td>
<td>145</td>
</tr>
</tbody>
</table>

IS calibrators were tested by two technologists over 20 days. Note that the lowest concentration (0.001% IS) had several negative wells resulting in a high SD and CV%.

CV%, percent coefficient of variation.

### Table 3: Analytical Performance Parameters of RT-ddPCR

<table>
<thead>
<tr>
<th>(N)</th>
<th>Target value (%)</th>
<th>Data mean</th>
<th>SD</th>
<th>CV%</th>
</tr>
</thead>
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<td>0</td>
<td>NA</td>
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<tr>
<td>6</td>
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<td>0.22354111</td>
<td>0.01722102</td>
<td>8</td>
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<tr>
<td>5</td>
<td>1</td>
<td>0.02718138</td>
<td>0.00393607</td>
<td>14</td>
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<td>6</td>
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<td>0.00021324</td>
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<tr>
<td>6</td>
<td>0.01</td>
<td>0.00025697</td>
<td>4.3107 × 10⁻⁵</td>
<td>16</td>
</tr>
<tr>
<td>6</td>
<td>0.001</td>
<td>2.0253 × 10⁻⁵</td>
<td>4.6423 × 10⁻⁶</td>
<td>23</td>
</tr>
<tr>
<td>6</td>
<td>0.0001</td>
<td>9.5105 × 10⁻⁶</td>
<td>1.0637 × 10⁻⁵</td>
<td>112</td>
</tr>
</tbody>
</table>

Total RNA was extracted from pooled patient samples and diluted in total RNA from a healthy donor. Replicates were tested together by two technologists over 20 days. Note that the lowest concentration (0.0001% IS) had several negative wells, resulting in a high SD and CV%.

CV%, percent coefficient of variation; NA, not applicable.
However, the method is semiquantitative because of the variable efficiency associated with the pre-amplification step.\textsuperscript{14} The pre-amplification step was necessary to lower the LOD (ie, improve analytical sensitivity). For our assay, the sample of a single well is partitioned into up to 20,000 droplets. The increased partitioning further increases the limits of detection and quantification, so pre-amplification is not necessary, and the test can be performed as a one-step RT-PCR. Adding the counts of additional wells can further improve LOD and LOQ.

Other investigators have demonstrated the advantages of quantifying genomic BCR-ABL1 DNA.\textsuperscript{32--44} The advantages as compared to conventional RT-PCR include improved LOD (1 to 2 log) and a more stable analyte that does not need to be normalized to the expression level of a housekeeping gene. However, the breakpoints for each patient are highly variable, requiring development and validation of primers and probes specific for each patient. This is technically challenging and labor intensive, and not easily adapted to the clinical laboratory. However, when rapidly sequencing and characterizing genomic BCR-ABL1 DNA becomes possible, ddPCR will have obvious advantages as compared to qPCR for monitoring disease burden. In addition to the improvement in accuracy and LOD/LOQ, limited only by the amount of patient material, development and validation of patient-specific assays will become easier because efficiency of amplification is less of a concern with ddPCR.

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