Development of an Integrated Assay for Detection of BCR-ABL RNA

Emily S. Winn-Deen,1* Bret Helton,1 Reuel Van Atta,1 Wendy Wong,1 Jeffrey Peralta,1 James Wang,1 Gregory J. Tsongalis,2 Dorothy Belloni,2 David Chan,2 James R. Eshleman,3 Christopher D. Gocke,3 Zsolt Jobbagy,3 Lan Beppu,4 and Jerald P. Radich4

Background: Current practice guidelines for managing patients with chronic myelogenous leukemia (CML) call for monitoring BCR-ABL transcript concentrations with a quantitative reverse transcription–PCR (qRT-PCR) assay. Because the available laboratory-developed assays lack consensus on the appropriate design, reporting of results, and reference intervals, we developed and evaluated an integrated BCR-ABL assay that yields standardized results for any laboratory and can be performed by technicians with no specialized training.

Methods: We used the Cepheid Xpert® BCR-ABL Monitor assay to measure both BCR-ABL and ABL (endogenous control) transcripts in blood samples from CML patients and healthy individuals. The assay involves 8 manual pipetting steps, fully automated nucleic acid purification, a nested qRT-PCR step, and data analysis.

Results: The BCR-ABL assay requires approximately 2 h 20 min and covers a 5-log concentration range with a lower detection limit for the BCR-ABL/ABL ratio of approximately 0.005%. Assay results were negative for 100% of the 56 known CML-negative samples (12 patients with other hematologic disorders and 44 healthy blood donors). Testing of CML-positive patients undergoing disease monitoring showed 85% agreement with negative results (17 of 20) and 100% agreement with positive results (26 of 26). An imprecision/portability study revealed no differences in performance between sites, days, instruments, and operators.

Conclusions: The Xpert BCR-ABL Monitor assay provides a robust and reproducible alternative to laboratory-developed assays. Its ease of use may allow more laboratories to offer BCR-ABL testing for patients, and the short assay time enables same-day results for treating physicians.

More than 95% of patients with chronic myelogenous leukemia (CML) possess the distinctive Philadelphia chromosome, which is produced by reciprocal translocation between the Abelson gene on chromosome 9 (ABL1) and the breakpoint cluster region gene of chromosome 22 (BCR). The resulting fusion gene (BCR-ABL) (1, 2), which has breakpoints at BCR exon b2 or exon b3 and at ABL exon a2, produces a p210 chimeric BCR-ABL tyrosine kinase with a deregulated activity that plays a key role in CML development (3).

The current practice guidelines of the Europe Against Cancer Program and the National Comprehensive Cancer Network recommend the use of quantitative reverse transcription–PCR (qRT-PCR) assays for managing CML patients (3–5). qRT-PCR is an accurate and highly sensitive method for detecting the BCR-ABL fusion gene (3–14) and is more sensitive than fluorescence in situ hybridization or cytogenetic analysis (3). Several groups have published advice on qRT-PCR assay design and interpretation (4, 5, 15–18).

We describe the use of the Cepheid GeneXpert® Dx System for BCR-ABL detection. This system automates and integrates sample purification, nucleic acid amplifi-

---

1 Cepheid, Sunnyvale, CA.
2 Dartmouth-Hitchcock Medical Center, Lebanon, NH.
3 Molecular Diagnostics Laboratory, Johns Hopkins Medical Institutions, Baltimore, MD.
4 Fred Hutchinson Cancer Research Center, Seattle, WA.
* Address correspondence to this author at: Cepheid, 904 Caribbean Dr., Sunnyvale, CA 94089-1189. Fax 408-541-6439; e-mail Emily.Winn-Deen@cepheid.com.

Received January 5, 2007; accepted July 3, 2007.
Previously published online at DOI: 10.1373/clinchem.2007.085472

---

5 Nonstandard abbreviations: CML, chronic myelogenous leukemia; LOD, limit of detection; qRT-PCR, quantitative reverse transcription–PCR; Ct, threshold cycle.
6 Human genes: ABL, ABL1, v-abl Abelson murine leukemia viral oncogene homolog 1; BCR, breakpoint cluster region.
cation, and detection of the target sequence in simple or complex samples with real-time PCR (19).

Materials and Methods

ASSAY DESIGN

The GeneXpert Dx System consists of an instrument and a personal computer preloaded with software for running tests and viewing results. Single-use GeneXpert cartridges hold assay reagents and host the sample-preparation and PCR processes. Because the cartridges are self-contained, cross-contamination concerns are eliminated.

Cepheid’s Xpert BCR-ABL Monitor assay is designed to detect the p210 BCR-ABL transcript with either the b2a2 or b3a2 breakpoint (Fig. 1). The BCR-ABL PCR amplicon spans the breakpoint between the BCR and the ABL sequences. After the initial RT-PCR step, the nested forward and reverse primers (Fig. 1) are used for a nested real-time PCR. The generated BCR-ABL amplicon has a length of either 145 bp (b3a2 breakpoint) or 77 bp (b2a2 breakpoint). The fluorogenic BCR-ABL probe hybridizes to the sense-strand sequence in the a2 region of the ABL gene near the BCR-ABL breakpoint (see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol53/issue9). We used standardized quantitated Armored RNA® samples (bacteriophage coat protein encapsulation of specific RNA targets to form pseudo-viral particles; Assuragen) for each breakpoint to verify that the assay design picked up both p210 targets equally.

The forward and reverse amplification primers for the endogenous ABL transcripts bind to the ABL sequence in the a2 and a3 regions, respectively (Fig. 1). Our design was to use the same reverse primer with a nested ABL forward primer in a seminested PCR for ABL to generate a 70-bp amplicon. The ABL fluorogenic probe hybridizes across the exon junction between exons a2 and a3 (see Table 1 in the online Data Supplement).

Only the 1st-round BCR-ABL forward and reverse PCR primers are adapted from the literature (13). A BLAST sequence query run against the human genome assembly showed no significant sequence homology between the BCR-ABL and ABL primers and probes and other sites in the human genome.

For the 1st PCR, we designed primer-annealing conditions so that the primers hybridized to both common alleles of the BCR exon 13 polymorphism (16). For the nested PCR, the primers perfectly match the amplicon sequence produced in the primary PCR. We also designed the BCR-ABL and ABL primers and probes so that they would not hybridize to the sequence encoding the ABL kinase mutation hotspot (20–24).

ASSAY PROCEDURE

The unit-dose cartridge and required liquid reagents are sold as single-use items in sets of 10 tests, and all components of the assay have been stabilized for storage at 4 °C. A 200-µL aliquot of whole blood is mixed with 40 µL proteinase K and 1 mL lysis reagent (Tris hydrochlo-

---

**Fig. 1.** Design of Cepheid’s Xpert BCR-ABL Monitor assay.

Locations of RT-PCR primers and nested primers are shown as green and orange arrows, respectively. A nested BCR-ABL amplicon of either 145 bp (b3a2 breakpoint) or 77 bp (b2a2 breakpoint) is generated depending on the translocation for the p210 target. The real-time TaqMan BCR-ABL probe (green) labeled with a 5’ reporter fluorophore (FAM) and a 3’ quencher (QSY7) hybridizes to the a2 region of the ABL gene near the BCR-ABL breakpoint. The TaqMan probe for ABL (orange) labeled with Texas Red (TxRd) reporter fluorophore at the 5’ end and a 3’ quencher (QSY21) hybridizes to the exon junction between exons a2 and a3.
ride, guanidine hydrochloride, pH 7.5) to inactivate nucleases and release nucleic acids from the cells. After a 10-min incubation at room temperature, 1 mL of pure ethanol is added to the lysed sample, and the mixture is transferred to the test cartridge. Wash, rinse, and elution reagents are then added to the designated ports in the test cartridge, the lid is closed, and the cartridge is loaded into the GeneXpert Dx System. The GeneXpert (a) isolates the total RNA from lysed whole blood by binding the RNA to the solid-phase purification material, (b) washes and rinses away inhibitors, (c) elutes the RNA in 75 μL buffer, (d) combines the RT-PCR reagent beads with the eluted RNA, (e) moves 25 μL of the sample-reagent mixture into the reaction tube, (f) performs quality checks to ensure that reagent preparation has been successful, (g) performs a 1-step RT-PCR followed by the primary PCR, (h) performs a nested real-time PCR protocol with a 20-fold dilution from the 1st reaction, (i) reviews the signal from both the ABL endogenous control and the BCR-ABL transcript for acceptability, and (j) calculates the difference in threshold cycle (ΔCt) between the 2 signals (see Tables 1 and 2 in the online Data Supplement for details on the primer/probe designs and PCR cycling conditions). The entire BCR-ABL test process requires approximately 2 h 20 min.

ASSAY QUALITY CONTROL

Cepheid generates a lot-specific calibration curve at the time of manufacture, and a certificate of analysis supplied with each reagent set contains the efficiency value for that reagent lot (see Fig. 1 in the online Data Supplement), which is used to calculate the ratio of BCR-ABL transcript to total ABL transcript in the tested patient’s sample. During the quality-control process, each new lot of reagents is tested for linearity and the limit of detection (LOD). If the predefined product-release specifications are met, Cepheid calculates a lot-specific efficiency value (EΔCt) from the slope of the calibration curve as:

\[ E_{ΔCt} = 10^{(1/slope)}, \]

where ΔCt is Ct(ABL) − Ct(BCR-ABL), a function of the concentration of K562 cell RNA in a background of normal RNA.

For a positive test result, the ratio of BCR-ABL transcript to ABL transcript is calculated as:

Percentage of BCR-ABL/ABL = (EΔCt) \( \Delta C_t \times 100. \)

For example, for a reagent lot with an EΔCt value of 1.915 and an assay with a ΔCt of −2.9, the ratio of BCR-ABL transcript to ABL transcript is: 1.915 − 2.9 × 100 = 15.2%. For assays with a valid ABL Ct and no detectable BCR-ABL signal, the user would first calculate a theoretical ΔCt by subtracting 32 (the upper-limit Ct cutoff value for BCR-ABL) from the assay’s actual ABL Ct value. For example, for a reagent lot with an EΔCt value of 1.915 and an assay with an ABL Ct of 15.4 and no measurable BCR-ABL Ct, the theoretical detection limit is calculated as follows:

\[ ΔC_t = 15.4 - 32 = -16.6; \]

\[ 1.915^{-16.6} \times 100 = 0.0021%. \]

The results are reported as BCR-ABL undetectable at a detection limit of 0.0021%.

PATIENT SAMPLES

Blood samples used in this work were obtained from healthy individuals with approval of the Stanford Medical Center institutional review board. Additional non-pathologic blood samples and other patient blood samples were the remainder of samples obtained for other purposes and were used according to the institutional review board oversight protocols of the respective authors’ institutions.

RESULTS

EFFECT OF ANTICOAGULANTS ON ASSAY PERFORMANCE

Blood drawn into heparin, EDTA, or citrate anticoagulants was used to study the effect of different anticoagulants on the assay. The 2 transcripts were comparably detected in samples containing citrate or EDTA blood anticoagulant. The use of heparin as an anticoagulant shifts the BCR-ABL signal to approximately 2–2.5 Ct values later, indicating some inhibition of the PCR. Low-end BCR-ABL “dropouts” or false negatives were also observed when heparin-containing samples were tested. On the basis of these results, we do not recommend the use of heparin-containing blood samples.

ASSAY SPECIFICITY

The specificity of the assay was tested with 44 nonpathologic citrated or EDTA-containing blood samples and a collection of 12 blood samples from patients with other hematologic disorders, including acute myelogenous leukemia, acute lymphocytic leukemia, Hodgkin lymphoma, multiple myeloma, and follicular lymphoma. Using the established BCR-ABL Ct cutoff value of 32, we found all these samples to be negative for BCR-ABL, for an overall specificity of 100%.

ASSAY CONCORDANCE

We tested a collection of 47 samples from CML patients in duplicate with both the Xpert assay and each laboratory’s current assay method at 3 clinical sites and reported the results for both the current assay method and the Xpert BCR-ABL Monitor assay. The Fred Hutchinson Cancer Research Center used their laboratory-developed assay for comparison (18), Johns Hopkins University ran a laboratory-developed assay based on the research-use-only reagent set purchased from Ipsogen, and Dartmouth sent their samples to an experienced external reference laboratory (Genzyme Genetics) for analysis. Because no
standardized BCR-ABL quantification standards are available for the normalization of results obtained with these different assay methods (16), the data were grouped into 3 ranges for analysis: negative (<0.01% BCR-ABL), low positive (0.01%–0.05% BCR-ABL), and high positive (>0.05% BCR-ABL). One sample could not be evaluated because the reference laboratory was not able to return a result. There was 85% agreement of our method with negative results obtained by the laboratories’ methods (17 of 20) and 100% agreement with positive results (26 of 26; Table 1). Duplicates were fit to an exponential curve by the least-squares method (ΔCt as the abscissa and %BCR-ABL as the ordinate). We analyzed residuals separately with 1-way ANOVA with the patient as the factor for each test site. The difference between the actual value and the predicted value from the ANOVA analysis is a measure of assay imprecision. We evaluated residuals for gaussian distribution with the Anderson–Darling test and for statistical control with an I-Chart to demonstrate statistical independence and control within the limits of having only duplicate values from each patient. Because these residuals are gaussian and independently distributed, we can compare the imprecision of the duplicates with the F-test. The final step in the analysis was to demonstrate that the assay imprecision limits around critical ΔCt values that were mapped onto the plane of the exponential curve did not overlap. This analysis indicated no statistically significant difference between duplicate measurements for the Xpert BCR-ABL Monitor assay for any of the patient samples tested.

ASSAY IMPRECISION

Assay imprecision was assessed in a multicenter (Fred Hutchinson Cancer Research Center, Dartmouth, Cepheid), blinded comparative study with pooled blood samples enriched with various concentrations of K562 RNA purified from the K562 leukemia cell line (which expresses the BCR-ABL fusion gene) and a single lot of Xpert BCR-ABL assay reagents. The enriched blood pools were lysed with Xpert BCR-ABL reagents, divided into aliquots, and shipped frozen to each site. Each site received 80 samples consisting of 20 samples for each of the 4 different RNA concentrations. Two technologists at each site participated in the 5-day study, with each technologist performing 2 runs of 4 samples each per day. Two of the sites (Fred Hutchinson Cancer Research Center, Dartmouth) used a novice operator who had had no experience with the Xpert BCR-ABL Monitor assay. Each run included 1 sample aliquot from each concentration. Percentage of BCR-ABL was calculated for each sample with the ΔCt value generated by the GeneXpert. Imprecision was estimated in accordance with the Clinical Laboratory Standards Institute guideline for evaluating imprecision performance of clinical chemistry devices (25). The 2 cartridges that failed to produce a result were omitted from the data analysis. Imprecision study results are summarized in Table 2. A nested ANOVA evaluation of each concentration indicated no statistically significant differences among operators, sites, instruments, or days. The 95% CIs around the mean values at each concentration tested indicated that the assay should be able to differentiate a 5-fold change in BCR-ABL transcript concentration, because the 95% CIs would not overlap.

ASSAY LINEARITY AND LOD

Linear range. To measure the linear range of the Xpert BCR-ABL Monitor test, we ran the assay with K562 RNA added to a nonpathologic blood sample (Fig. 2). At 500 ng and 5 μg K562 RNA, the ΔCt was close to zero, with the points falling below the line. At 50 pg K562 RNA (approximately 2 K562 cells (26)), all 6 replicates gave positive BCR-ABL results, and at 30 pg K562 RNA (approximately 1 K562 cell), 5 of the 6 replicates gave positive BCR-ABL results. At 10 pg K562 RNA (<1 K562 cell), only 3 of the 6 replicates gave positive BCR-ABL results, and at 1 pg K562 RNA, we detected no BCR-ABL signal in any of the 8 replicates tested. The Xpert BCR-ABL Monitor assay was linear between 50 pg and 50 ng K562 RNA (r² = 0.9939) and could detect BCR-ABL RNA over the 5-log range of 50 pg to 500 ng K562 RNA. Assay linearity for each analyte in the multiplex assay was also tested with a diagnostic patient sample with a leukocyte count of 4800/μL. The blood sample was diluted in duplicate by 10-fold serial dilutions into phosphate buffered saline (137 mmol/L NaCl, 10 mmol/L NaPO₄, 2.7 mmol/L KCl, pH 7.4) to yield 9.6 to 9.6 × 10⁴

<table>
<thead>
<tr>
<th>Test results</th>
<th>No. of CML samples</th>
<th>Site 1</th>
<th>Site 2</th>
<th>Site 3</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative by both methods</td>
<td></td>
<td>12</td>
<td>2</td>
<td>3</td>
<td>17</td>
</tr>
<tr>
<td>Negative by reference method, but low positive by Xpert BCR-ABL</td>
<td></td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Low positive by Xpert BCR-ABL and assay failure by reference method</td>
<td></td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Low positive by both methods</td>
<td></td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>High positive by both methods</td>
<td></td>
<td>10</td>
<td>2</td>
<td>6</td>
<td>18</td>
</tr>
</tbody>
</table>

* The comparison method at Fred Hutchinson Cancer Research Center (site 1) and Johns Hopkins University (site 3) was their laboratory-developed assay; Dartmouth (site 2) used the results for samples sent to a reference laboratory for comparison. Data were grouped into 3 categories: negative (<0.01% BCR-ABL), low positive (0.01%–0.05% BCR-ABL), and high positive (>0.05% BCR-ABL). There was 85% agreement with negative results (17 of 20) and 100% agreement with positive results (26 of 26) with the comparison method.
leukocytes. PBS without any cells was run in duplicate as the negative control. Both the BCR-ABL and the ABL signals were linear ($r^2 >0.98$) over a 5-log range of concentrations, and even the samples with approximately 10 cells per assay were detected by both assays (Fig. 3).

The slopes and intercepts for the BCR-ABL and ABL lines, which are a measure of assay efficiency, indicated that the assay measures both transcripts with nearly equal efficiency. On the basis of the established Ct limits for the ABL signal, this assay should yield valid results for blood

---

**Table 2. Analysis of assay imprecision in the multicenter, blinded comparative study.**

<table>
<thead>
<tr>
<th>Site</th>
<th>Sample</th>
<th>n</th>
<th>Mean BCR-ABL/ABL ratio, %</th>
<th>BCR-ABL/ABL ratio SD, %</th>
<th>95%, %</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Negative BCR-ABL</td>
<td>20</td>
<td>&lt;0.0001300013 (negative)</td>
<td>0.0056</td>
<td>0.0017–0.0078</td>
<td>118</td>
</tr>
<tr>
<td>2</td>
<td>Negative BCR-ABL</td>
<td>20</td>
<td>&lt;0.00018000018 (negative)</td>
<td>0.0034</td>
<td>0.00096–0.0047</td>
<td>118</td>
</tr>
<tr>
<td>3</td>
<td>Negative BCR-ABL</td>
<td>20</td>
<td>&lt;0.00016000016 (negative)</td>
<td>0.0062</td>
<td>0.0019–0.0087</td>
<td>118</td>
</tr>
<tr>
<td>Overall</td>
<td>Negative BCR-ABL</td>
<td>60</td>
<td>&lt;0.00016000016 (negative)</td>
<td>0.0052</td>
<td>0.0027–0.0059</td>
<td>122</td>
</tr>
<tr>
<td>1</td>
<td>Low BCR-ABL</td>
<td>20</td>
<td>0.0048</td>
<td>0.0052</td>
<td>0.0027–0.0059</td>
<td>122</td>
</tr>
<tr>
<td>2</td>
<td>Low BCR-ABL</td>
<td>19</td>
<td>0.0029</td>
<td>0.0034</td>
<td>0.00096–0.0047</td>
<td>118</td>
</tr>
<tr>
<td>3</td>
<td>Low BCR-ABL</td>
<td>20</td>
<td>0.0053</td>
<td>0.0062</td>
<td>0.0019–0.0087</td>
<td>118</td>
</tr>
<tr>
<td>Overall</td>
<td>Low BCR-ABL</td>
<td>59</td>
<td>0.0043</td>
<td>0.0052</td>
<td>0.0027–0.0059</td>
<td>122</td>
</tr>
<tr>
<td>1</td>
<td>Medium BCR-ABL</td>
<td>20</td>
<td>1.0</td>
<td>0.32</td>
<td>0.83–1.2</td>
<td>32</td>
</tr>
<tr>
<td>2</td>
<td>Medium BCR-ABL</td>
<td>20</td>
<td>0.86</td>
<td>0.43</td>
<td>0.62–1.1</td>
<td>50</td>
</tr>
<tr>
<td>3</td>
<td>Medium BCR-ABL</td>
<td>19</td>
<td>1.0</td>
<td>0.43</td>
<td>0.76–1.2</td>
<td>43</td>
</tr>
<tr>
<td>Overall</td>
<td>Medium BCR-ABL</td>
<td>59</td>
<td>0.95</td>
<td>0.40</td>
<td>0.84–1.1</td>
<td>42</td>
</tr>
<tr>
<td>1</td>
<td>High BCR-ABL</td>
<td>20</td>
<td>12</td>
<td>2.4</td>
<td>11–13</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>High BCR-ABL</td>
<td>20</td>
<td>12</td>
<td>7.3</td>
<td>8.3–16</td>
<td>60</td>
</tr>
<tr>
<td>3</td>
<td>High BCR-ABL</td>
<td>20</td>
<td>12</td>
<td>4.1</td>
<td>10–14</td>
<td>34</td>
</tr>
<tr>
<td>Overall</td>
<td>High BCR-ABL</td>
<td>60</td>
<td>12</td>
<td>5.0</td>
<td>11–14</td>
<td>41</td>
</tr>
</tbody>
</table>

*a Summary of results (cartridges that failed to produce a result were omitted from data analysis):
- Negative samples (no added K562 RNA): all tested as negative (range, <0.0001%–<0.0009%).
- Low-positive samples (100 pg K562 RNA): 56 of 59 tested as positive (range, 0.0002%–0.021%); 3 of 59 tested as negative.
- Medium-positive samples (5 ng K562 RNA): all tested as positive (range, 0.27%–2.0%).
- High-positive samples (100 ng K562 RNA): all tested as positive (range, 4.3%–37%).

*b Cepheid (site 1), Fred Hutchinson Cancer Research Center (site 2), Dartmouth (site 3).
*c BCR-ABL, BCR-ABL transcript.

---

Fig. 2. Assay linearity.

Linearity was tested with 1 pg, 10 pg, 30 pg, 50 pg, 500 pg, 5 ng, 50 ng, 500 ng, and 5 μg K562 RNA added to a nonpathologic blood sample. The ∆Ct between the BCR-ABL signal and the ABL signal was calculated at each K562 RNA concentration tested. TxR, Texas Red.
samples with leukocyte counts between 115/μL and 8000/μL.

**Lower LOD.** The lower LOD of the BCR-ABL/ABL transcript ratio depends on the difference between the amounts of the ABL and BCR-ABL transcripts in the sample. The valid Ct interval for the ABL signal is set at 12 < Ct < 18, whereas the valid interval for the BCR-ABL signal is set at 12 < Ct < 32. On the basis of these assay cutoff values, the theoretically best-case LOD occurs for an ABL Ct of 12 and a BCR-ABL Ct of 32 (i.e., ΔCt = −20). With a typical lot-specific efficiency value of 1.91, the best-case LOD would be a BCR-ABL/ABL ratio of 0.000240% (2 in 1 million). The theoretical worst-case LOD occurs for a ABL Ct of 18 and a BCR-ABL Ct of 32 (i.e., ΔCt = −14), for a worst-case LOD for the BCR-ABL/ABL ratio of 0.011629% (1 in 10 000). For the 44 nonpathologic EDTA-containing and citrated blood samples run in this study, the median ABL Ct was 14.5 (range, 12.0–17.5).

In laboratory experiments, the Xpert BCR-ABL Monitor assay detects as little as 50 pg of K562 RNA (approximately 2 cells; Fig. 2) or as few as 10 leukocytes (Fig. 3). In the multicenter imprecision study (Table 2), a non-pathologic blood sample enriched with 100 pg of K562 RNA (approximately 4 cells) per 200 μL anticoagulated whole blood and tested 59 times gave a mean BCR-ABL/ABL ratio of 0.0043% and was evaluated as positive for 56 (95%) of the 59 replicates. For the other 3 replicates, a BCR-ABL signal was detected, but it occurred after the BCR-ABL Ct cutoff value of 32. This result fits the conventional definition of the lower LOD, in which the sample is measured as positive 95% of the time. Similar results were reported in a separate evaluation by Johns Hopkins University scientists (27).

**Upper limit.** Samples containing very high leukocyte concentrations may exhibit an ABL signal that occurs below the Ct threshold value (<12) and/or may distort the

Fig. 3. Assay linearity for the multiplex BCR-ABL/ABL assay.
A blood sample was diluted into phosphate-buffered saline (137 mmol/L NaCl, 10 mmol/L NaPO4, 2.7 mmol/L KCl, pH 7.4) and assayed. The ABL signal (A) and the BCR-ABL signal (B) are plotted separately for clarity.
shape of the real-time amplification curve. If this occurs, the GeneXpert instrument displays the result as “Invalid”. To test the validity of using a smaller volume of blood when this error occurs, we tested 2 samples at various sample volumes. Both the BCR-ABL and ABL signals were reduced by almost a logarithm in processing a 10-μL sample but remained consistent with blood sample volumes of 25–200 μL. Samples containing very high leukocyte concentrations that display an “Error” or Invalid signal with a 200-μL sample can be repeated with less sample as long as the sample volume is at least 25 μL.

Discussion

The laboratory community concerned with measuring the BCR-ABL transcript has recognized for some time that not having a standardized testing method available creates patient care issues. The Europe Against Cancer Program has focused on selecting the most appropriate reference gene and on the design of standardized TaqMan assays (15, 17). A subsequent CML consensus conference further refined the recommendations for test samples, assay design, contamination prevention, and the reporting of results (16). The assay design rules expressed by the Europe Against Cancer Program (15, 17) and Hughes et al. (16) have been used as the model for the Xpert BCR-ABL Monitor assay. ABL has been selected as the control gene, and the ABL PCR assay has been designed to avoid the sequence encoding the ABL kinase domain, where mutations may emerge after imatinib treatment.

RNA instability during storage and transport can affect some qRT-PCR assay results. Typically, blood samples that are sent out for testing experience at least a 24-h delay between blood drawing and RNA isolation. Being simple enough to run in any molecular biology laboratory, the Xpert BCR-ABL Monitor assay can shorten the storage time between obtaining the blood sample and testing and may improve the detection of low concentrations of the BCR-ABL transcript, which is desirable when monitoring for minimal residual disease.

The assay can detect >5 logs of BCR-ABL transcript concentrations (Fig. 3). This performance is sufficient to detect a 3-log reduction from the BCR-ABL transcript concentration at diagnosis, and the CIs obtained in the imprecision study (Table 2) indicate that the assay is sufficiently precise to detect a 5-fold change in BCR-ABL transcript concentration over most of the assay range.

Current laboratory methods achieve their LOD by isolating RNA from milliliter quantities of whole blood. In contrast, the Xpert BCR-ABL assay uses a nested PCR with RNA isolated from only 200 μL of whole blood to achieve its LOD. Nested approaches are impractical to use in an open-assay system, but the closed nature of the Xpert BCR-ABL assay cartridge allows this method to be used routinely without fear of contaminating the laboratory with PCR amplicon. The closed system also allows the laboratory to carry out all assay steps in a single area, because all of the RNA-purification, reverse transcription, PCR, and detection steps are performed within the assay cartridge. There is no wet interface between the GeneXpert instrument and the assay cartridge, so there is also little danger of contamination of either the equipment or subsequent samples.

Current laboratory methods for detecting BCR-ABL transcript have many steps where errors can be introduced. To guard against these potential errors, investigators typically run their assays in duplicate or triplicate. In contrast, the Cepheid BCR-ABL Monitor assay has relatively few manual pipetting steps, all of which are quite insensitive to pipetting accuracy. The GeneXpert System performs most of the steps requiring accurate pipetting, thereby reducing pipetting error as well as chances for mix-ups or skipped reagent additions. The reproducibility of this assay (Table 2) and the statistical analysis of duplicates run with CML samples indicate that running replicate Xpert BCR-ABL assays may not be necessary to ensure confidence in the results.

Reporting of assay results has also been the subject of some debate. Reporting results as the ratio of the BCR-ABL transcript to the ABL transcript can factor out some of the between-laboratory variation (16). The Xpert BCR-ABL Monitor assay uses a lot-specific calibration curve to report results in the ratio format. Testing of each reagent lot against stringent quality-release criteria has demonstrated the calibration curve to have high between-lot reproducibility, and expiration dating of the assay has been set so that the curve does not change significantly over the shelf life of the product. This feature provides laboratory staff with the convenience of using lot-specific information without having to generate the calibration curve in their own laboratories. An effort is currently under way to further improve the reporting of results by providing testing laboratories with a standardized set of samples. These results would provide a conversion factor that could normalize results among laboratories to an international scale (16). This change would further standardize the BCR-ABL testing community and facilitate laboratory adoption of improved testing methods as they are developed.

In summary, the Xpert BCR-ABL assay provides a robust and reproducible alternative to laboratory-developed assays. Its ease of use may allow more laboratories to offer BCR-ABL testing to the patients they serve, and the short assay time (2 h 20 min) enables same-day results for treating physicians. Such rapid sample turnaround can reduce patient anxiety if the results are good and permit more rapid adjustment of treatment if the results indicate an increase in BCR-ABL concentration.

Grant/funding support: E.S.W.-D., B.H., R.V.A., W.W., J.P., and J.W. are employed by Cepheid, whose product was studied in the present work. Cepheid supplied the GeneX-
pert instrument and BCR-ABL reagents to the other 3 institutions participating in this study.

Financial disclosures: None declared.

Acknowledgments: We thank Joseph Whitmore of Cepheid for the statistical analysis of assay imprecision.

References


