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Quality Control Methods for Optimal BCR-ABL1 Clinical Testing in Human Whole Blood Samples

Lauren M. Stanoszek, Erin L. Crawford, Thomas M. Blomquist, Jessica A. Warns, Paige F.S. Willey, and James C. Willey

From the Department of Medicine, University of Toledo Health Sciences Campus, Toledo, Ohio

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Address correspondence to James C. Willey, M.D., Division of Pulmonary and Critical Care Medicine, Department of Medicine, Room 0012 Ruppert Health Bldg, University of Toledo Health Sciences Campus, 3000 Arlington Ave, Toledo, OH 43614. E-mail: James.Willey2@utoledo.edu.

Reliable breakpoint cluster region (BCR)—Abelson (ABL) 1 measurement is essential for optimal management of chronic myelogenous leukemia. There is a need to optimize quality control, sensitivity, and reliability of methods used to measure a major molecular response and/or treatment failure. The effects of room temperature storage time, different primers, and RNA input in the reverse transcription (RT) reaction on BCR-ABL1 and β-glucuronidase (GUSB) cDNA yield were assessed in whole blood samples mixed with K562 cells. BCR-ABL1 was measured relative to GUSB to control for sample loading, and each gene was measured relative to known numbers of respective internal standard molecules to control for variation in quality and quantity of reagents, thermal cycler conditions, and presence of PCR inhibitors. Clinical sample and reference material measurements with this test were concordant with results reported by other laboratories. BCR-ABL1 per 10^3 GUSB values were significantly reduced ($P = 0.004$) after 48-hour storage. Gene-specific primers yielded more BCR-ABL1 cDNA than random hexamers at each RNA input. In addition, increasing RNA inhibited the RT reaction with random hexamers but not with gene-specific primers. Consequently, the yield of BCR-ABL1 was higher with gene-specific RT primers at all RNA inputs tested, increasing to as much as 158-fold. We conclude that optimal measurement of BCR-ABL1 per 10^3 GUSB in whole blood is obtained when gene-specific primers are used in RT and samples are analyzed within 24 hours after blood collection. *(J Mol Diagn 2013, 15: 391—400; http://dx.doi.org/10.1016/j.jmoldx.2013.02.004)*

Chronic myelogenous leukemia (CML) represents 15% of all adult leukemias in Western populations.[1] This malignancy is caused by a chromosomal translocation, t(9:22)(q34;q11); a segment of the breakpoint cluster region (*BCR*) gene from chromosome 22 (region q11) is fused to a site within the Abelson (*ABL1*) gene from chromosome 9 (region q34).[2,3] Most BCR breakpoints occur within exons e12 to e16 (b1 to b5; alias major breakpoint cluster region) and fuse to ABL1 exon 2 breakpoints, resulting in e13a2 (b2a2) or e14a2 (b3a2) fusion transcripts that encode 210-kDa proteins.[4] The BCR-ABL1 fusion protein is a constitutively active tyrosine kinase responsible for the uncontrolled proliferation observed in CML.[5–7] For the past decade, first-line therapy for chronic-phase CML has been imatinib mesylate (Novartis Pharmaceuticals, Basel, Switzerland), which inhibits the tyrosine kinase activity of the BCR-ABL1 protein.[8,9] Imatinib mesylate binds to the ATP-binding pocket of the BCR-ABL1 fusion protein, stabilizing it in its inactive form.[10] Although second-generation tyrosine kinase inhibitors, such as dasatinib (Bristol-Myers Squibb Company, Princeton, NJ) and nilotinib (Novartis Pharmaceuticals), have been used in imatinib-resistant or intolerant patients with CML, recent studies show promise in these agents as more potent first-line therapies for those newly diagnosed with chronic-phase CML.[11–16]

After treatment, a major molecular response (MMR) is defined, based on results from the International Randomized Interferon versus STI-571 study,[17] as a 3-$\log_{10}$ reduction from a standardized median baseline value in the level of measured BCR-ABL1 transcript. In the International Randomized Interferon versus STI-571 study, patients with a BCR-ABL1 value at or lower than the MMR within 12 to 18 months of

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beginning imatinib treatment were 100% free from accelerated-phase or blast crisis at 5 years.[18] A subsequent 0.5-$\log_{10}$ increase in BCR-ABL1 transcript abundance from treated baseline indicates treatment resistance and the need to begin a second-generation tyrosine kinase inhibitor or plan for allogeneic stem cell transplantation.[19–21] National Comprehensive Cancer Network Guidelines recommend BCR-ABL1 monitoring every 3 months to provide fusion transcript-level trend data.[19,20,22] Timely BCR-ABL1 monitoring is essential to identify patients who are unresponsive to therapy in the early phase of treatment. Therefore, an accurate baseline or normalized value of BCR-ABL1 fusion transcript abundance must be obtained at the time of diagnosis and subsequent BCR-ABL1 levels must be accurately measured to monitor therapeutic efficacy over time.[18]

Several quantitative RT-PCR–based BCR-ABL1 diagnostic tests are commercially available. In addition, standardized methods have been developed for clinical laboratory measurement of BCR-ABL1 in whole blood,[4,22,23] including use of the International Scale for interlaboratory harmonization.[24–29] The International Randomized Interferon versus STI-571 study standardized diagnostic baseline is defined as 100% International Scale value, and MMR is defined as 0.1% International Scale value.[30] BCR-ABL1 measurement results are converted to the International Scale via use of the World Health Organization panel of reference reagents with designated BCR-ABL1 International Scale values.[31,32]

An element missing from existing BCR-ABL1 tests is adequate control for intersample variation in PCR-interfering substances and interreaction variation in quality and quantity of PCR reagents or thermal cycling efficiency. These problems could cause inaccurate and possibly false-negative results. To address this, in the method described herein, BCR-ABL1 and β-glucuronidase (GUSB) were each measured relative to a known number of respective synthetic internal standard (IS) molecules.

Another potential source of inconsistency in BCR-ABL1 measurement is variation in the efficiency of mRNA-to-cDNA conversion during reverse transcription (RT). Blood-specific inhibitors of RT may be present within RNA extracted from whole blood, including heme, IgG, leukocyte genomic DNA, and the anticoagulants, EDTA and heparin.[33–37] RNA extracted from whole blood is mostly from leukocytes; however, a fraction of the RNA came from reticulocytes. Reticulocytes contribute an abundance of interfering α- and β-globin mRNA that may compete with lowly expressed transcripts, such as BCR-ABL1 for reagents within an RT reaction.[38,39] Reverse transcription efficiency is also dependent on the priming method used.

Clinical laboratories that conduct BCR-ABL1 molecular monitoring generally analyze whole blood samples collected in tubes containing EDTA to prevent coagulation-associated cytolysis. However, even in such tubes, relative representation of certain genes may change as early as minutes after venipuncture because of an altered regulation and/or degradation rate.[40,41] Thus, there is a need to identify the optimal RT priming method for both BCR-ABL1 and the reference gene, *GUSB*, under the most optimal storage time. The optimal amount of whole blood RNA to include in the RT reaction may be affected by each of the previously mentioned factors.

In an effort to establish optimal conditions for measurement of BCR-ABL1, we did the following: i) developed a quality-controlled, quantitative PCR method, ii) measured the effect of total RNA input or type of RT primers on RT efficiency, and iii) assessed the effect of storage time on BCR-ABL1 measurement.

Materials and Methods

Samples from CAP MRD-B—BCR-ABL1 p210 2011 Proficiency Survey

Three proficiency samples were obtained from the College of American Pathologists (CAP; Northfield, IL). Sample minimal residual disease (MRD)-04 was RNA extracted from the K562 cell line that expresses the b3a2 BCR-ABL1 transcript. Sample MRD-05 was RNA extracted from a BCR-ABL1–negative cell line. Sample MRD-06 was RNA extracted from MRD-04 diluted 1:10,000 in BCR-ABL1–negative RNA (MRD-05).[42] Each RNA sample was DNase treated with DNA-*free* DNAse Treatment and Removal Reagent (Life Technologies, Grand Island, NY).

Blood Samples

All blood samples were collected under protocols approved by the University of Toledo (Toledo, OH) Medical Center Institutional Review Board. Whole blood samples were collected in 4-mL dipotassium EDTA Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) at ambient temperature at the University of Toledo Medical Center. Blood used to mix with K562 cells (described later) was obtained through the Department of Pathology, University of Toledo Medical Center, from patients undergoing routine venipuncture for laboratory testing. Leftover blood not needed for standard-of-care purposes from patients with a normal complete blood cell count was anonymized and provided to the research laboratory. Peripheral blood was obtained from three patients with CML [two with chronic-phase (CP) CML newly diagnosed and one with CP-CML at MMR under treatment] at the same time blood was drawn for shipment to ARUP Laboratories (Salt Lake City, UT) for BCR-ABL1 measurement and conversion of results to the International Scale.

Samples for Kinetic Stability Study

The K562 cell line, expressing the BCR-ABL1 b3a2 fusion transcript, was purchased from ATCC (Manassas, VA) and incubated in RPMI 1640 medium + 10% fetal bovine serum at 37°C, in an atmosphere containing 5% CO_2 and 90%

humidity. K562 cells were isolated from culture at 80% confluency. In each of the two experiments, whole blood samples were collected in EDTA tubes from three anonymized individuals, and each sample was spiked with a known number of K562 cells suspended in PBS. In the first experiment (samples A, B, and C), the concentration of K562 cells in each whole blood sample was 5.8×10^5 cells/mL (2.32×10^6 cells total), and in the second experiment (samples D, E, and F), the concentration in each sample was 4.7×10^6 K562 cells/mL (1.88×10^7 cells total). Each whole blood/K562 cell sample was incubated in 15-mL conical tubes at room temperature for various amounts of time. For each sample, the time course was initiated within 6 hours of venipuncture. At each time point, individual conical tubes were inverted three times before RNA extraction.

Sample for Comparison of Three RT Priming Methods Study

Known numbers of K562 cells were spiked into anonymized whole blood samples collected in EDTA tubes from one anonymized individual (K). The concentration of K562 cells in sample K was 5.8×10^5 cells/mL (2.32×10^6 cells total).

Samples for RNA Input versus RT Priming Method Study

Known numbers of K562 cells were spiked into anonymized whole blood samples collected in EDTA tubes from each of three individuals (G, H, and I) for the random hexamer (RH)–primed RT efficiency study. The concentration of K562 cells was 4.4×10^6 K562 cells/mL (1.76×10^7 cells total) in samples G and H and 5.0×10^6 K562 cells/mL (2.00×10^7 cells total) in sample I. For the gene-specific primed RT efficiency study, blood from one anonymized individual (J) was used. The concentration of K562 cells was 4.9×10^6 K562 cells/mL (1.76×10^7 cells total).

Preparation of RTSM

In vitro–transcribed synthetic, alien RNA standards developed by the External RNA Control Consortium (ERCC), termed ERCC 171 and 113, were donated by Dr. Marc Salit, National Institute for Standards and Technology (Gaithersburg, MD).[43–45] RNA stocks from ERCC 113 and 171 standards were first diluted in RNase-free water, and the RNA concentration was measured using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). ERCC 113 and 171 standards were each secondarily diluted in 100 ng/μL salmon sperm DNA to a final concentration of 1.0 (10^{-10}) mol/L (Invitrogen, Carlsbad, CA). The ERCC 113 standard was reverse transcribed into cDNA, and the cDNA was quantified using the Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA). A reverse transcription standards mixture (RTSM) was prepared by combining known molar amounts of ERCC 171 RNA and ERCC 113 cDNA (previously reverse transcribed). A 1-μL aliquot of RTSM was included in each RT reaction to assess RT efficiency. The ERCC 171 RNA was reverse transcribed into cDNA, along with other RNA species in the RT reaction, whereas the ERCC 113 cDNA remained unchanged. Yield of ERCC 171 cDNA was a measure of RT efficiency, and ERCC 113 cDNA was included as a sample loading control. Each 1-μL aliquot of RTSM contained 1.42×10^4 ($\pm 6.7 \times 10^3$) ERCC 171 cDNA molecules and 1.68×10^4 ($\pm 7.4 \times 10^3$) ERCC 113 cDNA molecules when measured in RT reactions with no background RNA.

Measurement of RT Efficiency

After RT, an aliquot of RT product, cDNA, was subjected to PCR in the presence of a known number of IS molecules for ERCC 171 and ERCC 113. ERCC 171 RNA RT efficiency was determined by measuring the ERCC 171 cDNA native template (NT)/IS PCR product ratio over the ERCC 113 NT/IS product ratio. In this way, normalization of ERCC 171 cDNA molecules to ERCC 113 cDNA molecules controlled for variation in RTSM sample loading, enabling reliable measurement of RT efficiency.

Sequencing of Background Peaks

PCR products associated with the background noise peaks were isolated using an E-Gel SizeSelect 2% gel with the E-Gel iBase Power System (Invitrogen), and tailed BCR-ABL1 b3a2 sequencing primers corresponding to Ion Torrent Amplicon Sequencing adapters were used to amplify both isolated native and off-target products (Ion Torrent Amplicon Application Note, April 4, 2011; Amplicon Sequencing, South San Francisco, CA). Amplified products with attached Ion Torrent Amplicon Sequencing adapters were gel purified using E-Gel Size Select 2% and sent to the Ohio University Genomics Facility for Ion Torrent 314 DNA Chip Sequencing Service (Ohio University, Athens, OH). Sequencing data captured internally to the sequencing reagents were used to build consensus sequences using ClustalX 2.1 (Conway Institute UCD, Dublin, Ireland).[46] These consensus sequences were then subjected to BLAST search of the National Center for Biotechnology Information human genomic and transcript databases (*http://blast.ncbi.nlm.nih.gov/Blast.cgi*, last accessed December 18, 2012).

RNA Extraction

RNA was extracted with the QIAamp RNA Blood Mini Kit provided by QIAGEN (Hilden, Germany), according to the manufacturer's protocol. Each QiaAmp spin-column can prepare up to 1.5 mL of human whole blood. Each RNA sample was eluted with RNase-free water. In the kinetic cytolysis and RT primer comparison studies, RNA was extracted from single 1-mL anonymized whole blood/K562 samples per individual. In the RNA input versus RT

efficiency study, RNA was extracted from four separate spin columns (1 mL each) with the same eluate volume of RNase-free water applied to each of the four columns.

For the low BCR-ABL1 RNA RT priming comparison, patient blood sample RNA with undetectable BCR-ABL1 levels was extracted using the QiaAmp RNA Blood Mini Kit (QIAGEN). Extracted patient blood RNA was mixed with a 0.6-μL K562 cell RNA (5.66 ng/μL) that was extracted using TriREAGENT (Molecular Research Center, Inc., Cincinnati, OH).

For all samples, RNA concentration and purity were each measured using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). RNA integrity was evaluated on an Agilent 2100 Bioanalyzer for quantification and characterization of 28s and 18s ribosomal RNA bands. Agilent software version B.02.03.SI307 calculated an RNA integrity number (RIN) for each sample assessed. RNA samples were stored at −20°C.

Reverse Transcription

All total RNA samples were reverse transcribed using the Superscript III First Strand Synthesis System for RT-PCR (Invitrogen). A 30-μL RT reaction volume was used and contained 0.5 μg total RNA (kinetic stability study), various RNA inputs, including 0.9, 3, 5, 9, and 18 μg (RT input study), or 1.0 μg total RNA (RT priming comparison study). Reverse transcription reaction volumes were increased for experiments, including the CAP Proficiency Sample Study (75 μL) and the CML Patient Study (subject 806, 40 μL; and subjects 807 and 808, 60 μL). In a 30-μL RT reaction, sample RNA in 12 μL RNase-free water was denatured by 5-minute incubation at 94°C and then placed on ice to cool. An 18-μL RT master mix was then added, and the RT reaction mixture was incubated at 50°C for 1 hour, followed by 5-minute incubation at 94°C. The RT master mix included the following: 10× RT buffer, 25 mmol/L $MgCl_2$, 0.1 mol/L dithiothreitol, 10 mmol/L dNTP mix, 40 U/μL RNaseOUT, and 200 U/μL SuperScript III RT with 25 μmol/L RH, 50 μmol/L oligo (d)T primers, or 3 μmol/L gene-specific primers (Invitrogen). The PCR reverse primer was used as the gene-specific RT primer for GUSB, whereas BCR-ABL1 gene-specific RT primers included either the PCR reverse primer (19 bases) or an extended primer (29 bases).

Transcript Abundance Measurement

Sample loading was controlled by measuring BCR-ABL1 relative to GUSB.[47–49] According to the standardized RT-PCR method, BCR-ABL1 and loading control gene, *GUSB*, were each measured relative to a known number of their respective IS molecules within an Internal Standards Mixture (ISM).[50,51] The ISM used for these studies was purchased from Accugenomics, Inc. (Wilmington, NC). Transcript abundance values were then calculated as BCR-ABL1 molecules per 10^3 GUSB molecules. The competitive template IS for BCR-ABL1 and GUSB were each 10% to 20% shorter than the target gene NT PCR product, but both were amplified with the same efficiency by the same pair of primers. BCR-ABL1 b3a2 and b2a2 fusion transcript cDNAs were measured with the same BCR-ABL gene-specific PCR forward and reverse primer and measured relative to the same IS sequence. BCR-ABL1 b3a2 and b2a2 PCR products were different lengths that could be electrophoretically separated. Sequence information for primers and IS is provided in Table 1. The presence of an IS controlled for sample loading, intersample variation in the presence of PCR inhibitors (which often are gene specific), and ensured no false negatives (if the PCR failed, the IS PCR product was not observed and there were no data to report).[51] False positives due to contamination were eliminated through use of a control PCR sample with neither native nor competitive template.

Before amplification, cDNA and ISM were combined into a master mix, along with the appropriate volume of RNase-free H_2O, 30 mmol/L 10× buffer containing $MgCl_2$ (Idaho Technology, Salt Lake City, UT), 2 mmol/L dNTPs

Table 1 Primer and IS Sequences

	Primers			
Target	Forward	Reverse	NT length	IS length
BCR-ABL1 b3a2 (b2a2)	5′-ACTCCAGACTGTCCACAGCA-3′	5′-TTGGGGTCATTTTCACTGG-3′	231 (164)	123
GUSB	5′-GACTGAACAGTCACCGACGA-3′	5′-GTAAACGGGCTGTTTTCCAA-3′	174	139
ERCC 113	5′-TTGGATCAGTGGGAAGTGCT-3′	5′-GGGGCTCGAAAGGTACTAGG-3′	130	102
ERCC 171	5′-AAGCTGACGGTGACAAGGTT-3′	5′-TCGCAGTTTTCCTCAAATCC-3′	118	97
	Internal Standard			
BCR-ABL1 b3a2 (and b2a2)	5′-ACTCCAGACTGTCCACAGCATTCCGCTGACCATCAATAAGGAAGAAGCCCTTCAGCGGCCAGTAGCATCTG ACTTTGAGCCTCAGGGTCTGAGTGAAGCCGCTCCCAGTGAAAATGACCCCAA-3′			
GUSB	5′-GACTGAACAGTCACCGACGAGAGTGCTGGGGAATAAAAAGGGGATCTTCACTCGGCAGAGACAACCAAAAAGT GCAGCGTTCCTTTTGCGAGAGAGATACTGGAAGATTGCCAATGAAATTGGAAAACAGCCCGTTTAC-3′			
ERCC 113	5′-TTGGATCAGTGGGAAGTGCTCACGCGCGGAGCCCACTGGGCGAACAGCAACGTTATAACGGCCACTCAGTG GTTCGTCACGCCCTAGTACCTTTCGAGCCCC-3′			
ERCC 171	5′-AAGCTGACGGTGACAAGGTTTCCCCCTAATCGAGACGCTGCAATAACACAGGGGCATACAGTAACCAGGCA AGAGTTGGATTTGAGGAAAACTGCGAAAAA-3′			

(Promega, Madison, WI), 50 ng/μL gene-specific primers, and a minimum of 0.5 U Taq polymerase (Promega). For PCR amplification of blood/K562 cDNA samples that had been reverse transcribed with the 29-base BCR-ABL1 RT gene-specific primer, a minimum of 0.5 U of Gotaq Hotstart polymerase (Promega) was used. For each experiment, sufficient master mixture was prepared to measure each of the desired genes. This mixture was divided into tubes containing primers for single genes. All PCRs were performed in a Rapidcycler (Idaho Technology) for 35 cycles. All reactions were denatured for 5 seconds at 94°C (2 minutes at 94°C for Gotaq Hotstart polymerase in the first cycle), annealed for 10 seconds at 58°C, and elongated for 15 seconds at 72°C. After PCR amplification, the IS and NT for each gene were electrophoretically separated and quantified on an Agilent 2100 Bioanalyzer using DNA chips with DNA 1000 kit reagents for visualization, according to the manufacturer's protocol (Agilent Technologies Deutschland GmbH, Waldbronn, Germany). Area under the curve values for the NT and IS electrophoretic peaks for each gene were used for gene expression measurement.

Statistical Analysis

Each measurement was performed in at least triplicate. Results were expressed as means ± SD. Statistical significance was calculated using a Student's *t*-test from Data Analysis Tools in Microsoft Office Excel (Microsoft Corporation, Redmond, WA), and figures were generated with GraphPad Prism version 6 (GraphPad Software Inc., La Jolla, CA). The differences were considered significant if $P < 0.05$.

Results

CAP MRD-B—BCR-ABL1 p210 2011 Proficiency Survey Sample Study

BCR-ABL1 b3a2 per 10^3 GUSB values were measured in the three CAP proficiency samples. As expected, BCR-ABL1 b3a2 was not detected in BCR-ABL1—negative sample MRD-05. Compared with baseline sample MRD-04, BCR-ABL1 per 10^3 GUSB was 3.55-$\log_{10}$ lower in sample MRD-06 (Figure 1A). As indicated in the figure, this result was not significantly different from the mean difference measured by other laboratories in the survey.

Patient Sample Comparison between BCR-ABL1 per 10^3 GUSB and International Scale Values

Changes in BCR-ABL1 per 10^3 GUSB values were closely correlated with changes in values measured by ARUP Laboratories for the three CML patient samples. ARUP Laboratories measured BCR-ABL1 relative to ABL1 and converted values to the International Scale. A linear trendline fit BCR-ABL1 per 10^3 GUSB to International Scale data points with high correlation ($R^2 = 0.99$) (Figure 1B).

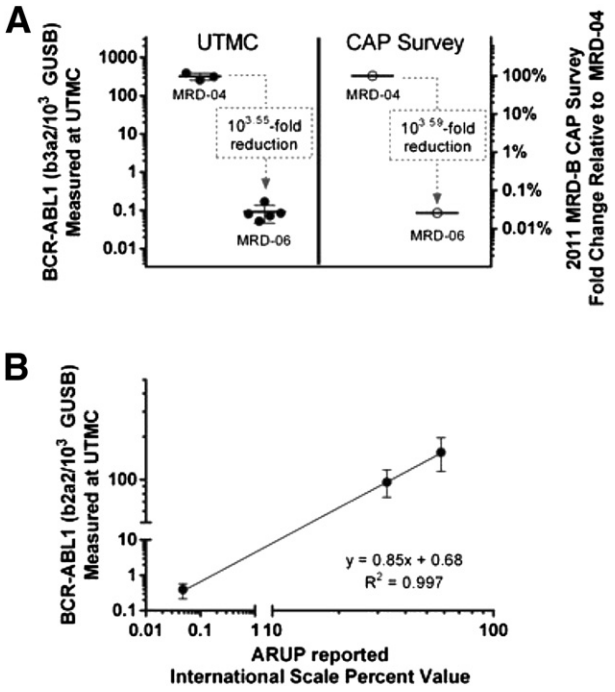

Figure 1 Interlaboratory comparison of BCR-ABL1 measurement. **A:** $\log_{10}$ reduction of BCR-ABL1 per 10^3 GUSB measurement in CAP MRD-B—BCR/ABL1 p210 2011 Survey measured at the University of Toledo Medical Center (UTMC) compared with median $\log_{10}$ reduction values of survey participants. **B:** Comparison of BCR-ABL1 per 10^3 GUSB measurement to International Scale percentage values reported by ARUP Laboratories in three CML patient blood samples (two with CP-CML newly diagnosed without treatment and one with CP-CML at MMR under treatment). Gene-specific RT primers were used for newly diagnosed CML patient samples, and RH RT primers were used for the MMR CML patient sample. The UTMC data presented are means for three or more replicate measurements. Error bars, SD of the means.

Effect of Incubation at Room Temperature on BCR-ABL1 per 10^3 GUSB Values

BCR-ABL1 per 10^3 GUSB transcript abundance and RIN score were measured at each time point and plotted as change relative to baseline (undegraded) values (Figure 2). When data from six anonymized individuals were combined, the BCR-ABL1 per 10^3 GUSB mean value trended down at 24 hours but was not significantly decreased until 48 hours (51% decrease, $P = 0.004$). Relative to baseline, the RIN score remained unchanged across all time points measured.

RT Primer Effect on Yield of BCR-ABL1 b3a2 or GUSB

We compared oligo (d)T, RH, and gene-specific RT primers to determine which gave the greatest yield of BCR-ABL1 and GUSB cDNA (Figure 3A). For both BCR-ABL1 b3a2 and GUSB, with RNA input into the RT reaction held constant (1 μg RNA/RT), the highest yield of cDNA was obtained with the use of gene-specific primers in the RT reaction. The yield with gene-specific primers was 17-fold ($P = 0.0001$) and 21-fold ($P = 0.0008$) higher for BCR-ABL1 b3a2 and GUSB, respectively, compared with RH (Figure 3A). Because the yield of each gene increased about the same amount,

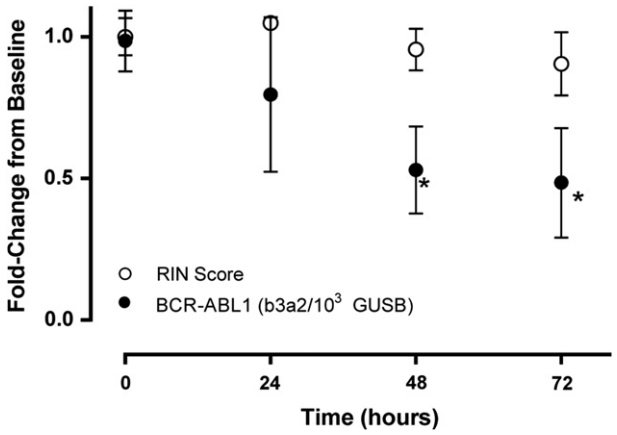

Figure 2 Effect of storage time on BCR-ABL1 measurement and RIN score. Effect of incubation at room temperature on BCR-ABL1 per 10^3 GUSB measurement and RIN score value relative to baseline time equal to 0 hours. RNA was extracted from whole blood collected in EDTA tubes, mixed with K562 cells, and then incubated at room temperature for 3 days. The random hexamers were used for reverse transcription. **Asterisks** denote statistical significance ($P < 0.01$) from baseline measurement at 0 hours. The results are means for samples from six donors, with three or more replicate measurements of each sample.

normalized (BCR-ABL1 b3a2 per 10^3 GUSB) values, measured with RH versus gene-specific RT primers, were not significantly different. In contrast, the BCR-ABL1 yield with oligo (d)T primers was fourfold lower ($P = 0.0007$) and GUSB cDNA yield was 17-fold higher ($P = 0.0001$) with oligo (d)T compared with RH primers (Figure 3A). Because the effect was different on BCR-ABL1 and GUSB, BCR-ABL1 b3a2 per 10^3 GUSB values were significantly different compared with those from RH or gene-specific primers ($P = 0.0001$).

Effect of RNA Input and RT Primer on Yield of BCR-ABL1 b3a2 or GUSB cDNA

When RH primers were used in RT, a maximum threefold increase ($P = 0.003$) in BCR-ABL1 b3a2 (molecules/μL cDNA) was observed at 18 μg RNA/RT compared with baseline at 0.9 μg RNA/RT. Because the slope for GUSB was not significantly different from that for BCR-ABL1 ($P \geq 0.35$), there was no significant difference among the BCR-ABL1 b3a2 per 10^3 GUSB values for each level of RNA input (Figure 3B).

The effect of increasing RNA input into RT reactions using gene-specific RT priming was also tested. Yields of BCR-ABL1 and GUSB molecules/μL cDNA were 17- and 20-fold higher, respectively, at baseline 0.9 μg RNA/RT reaction (30 ng RNA/μL cDNA) compared with samples primed with RH (Figure 3B). Consequently, with gene-specific primers, at 18 μg RNA/RT reaction (600 ng RNA/μL cDNA), BCR-ABL1 and GUSB yields were 158- and 312-fold higher, respectively, with gene-specific RT priming compared with RH RT priming. As with the cDNA from RH RT, because the slope for GUSB was not significantly different from that for BCR-ABL1 ($P \geq 0.35$), there was no significant difference among the BCR-ABL1 b3a2 per 10^3 GUSB values for each level of RNA input.

RNA Input Effect on RT Efficiency with RH (ERCC 171/113)

The RTSM (as described in *Materials and Methods*) was used to directly measure whether reduced cDNA yield with

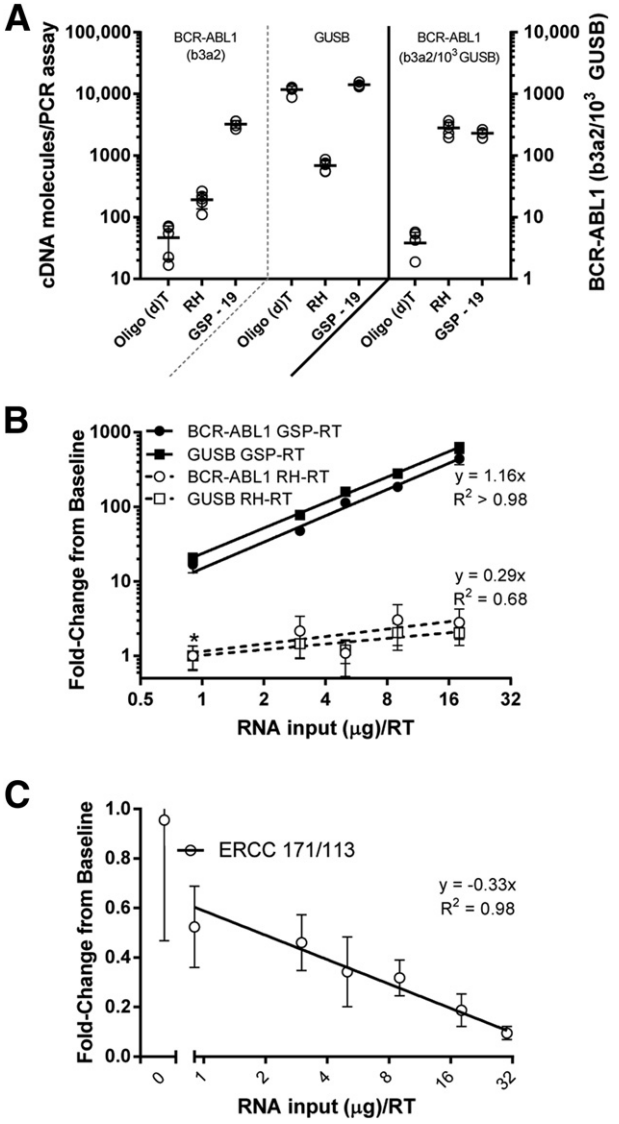

Figure 3 Effect of RT primer and RNA input on measured transcript abundance. **A:** Effect of RT priming method on yield of BCR-ABL1 b3a2 and GUSB molecules/PCR assay and BCR-ABL1 b3a2 per 10^3 GUSB measurement after quantitative RT-PCR of RNA extracted from whole blood/K562 mixture with high level of BCR-ABL1 transcript. GSP-19, gene-specific (19-base) primer. **B:** Effect of blood/K562 cell mixture RNA concentration in a 30-μL RT reaction and RT priming method on yield of BCR-ABL1 b3a2 or GUSB cDNA relative to RH-primed baseline (0.9 μg RNA/RT). **Asterisk** denotes baseline measurements on graph from which fold-changes were determined. GSP-RT is gene-specific primed RT reaction, and RH-RT is RH-primed RT reaction. **C:** Effect of RNA input on RH-primed RT efficiency. RT efficiency is ERCC 171/113 measurement normalized to ERCC 171/113 measurement for 0 μg RNA input. The results are means for samples with three or more replicate measurements of each sample. Error bars, SD of the means.

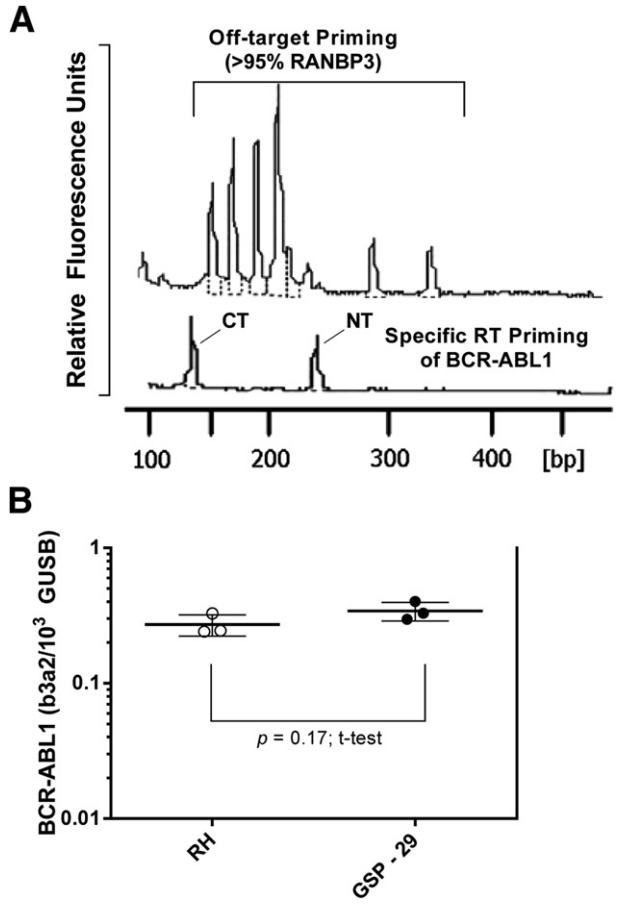

Figure 4 Effect of extended BCR-ABL1 gene-specific RT primer on target specificity. **A:** Agilent Bioanalyzer electropherograms demonstrating (**top line**) off-target reverse transcription priming with 19-base BCR-ABL1 gene-specific RT primer with sample having low level BCR-ABL1. NT and IS product peaks are outcompeted in PCR by RANBP3 transcript isoforms (**bottom line**), presence of clean NT and IS peaks with 29-base BCR-ABL1 gene-specific RT primer. **B:** Effect of RT priming method on BCR-ABL1 b3a2 per 10^3 GUSB measurement after reverse transcription of low level of BCR-ABL1 transcript in a blood background. GSP-29, gene-specific primer (29 bases). The data presented are means for three or more replicate measurements. Error bars, SD of the means.

RH was the result of RT inhibition. Compared with baseline (0 μg background RNA/RT), ERCC 171/113 RT efficiency with RH primers was reduced by 52% ($P = 0.02$) at 3 μg RNA/RT (100 ng RNA/μL cDNA) and reduced by 80% ($P = 0.002$) at 18 μg RNA/RT (600 ng RNA/μL cDNA). A semilog plot trend line fits the data points with high correlation ($R^2 = 0.98$) (Figure 3C). ERCC 171/113 values were measured in the same RH-primed cDNAs used in Figure 3B.

Extended BCR-ABL1 Gene-Specific RT Primer Effect on Yield of BCR-ABL1 per 10^3 GUSB Values

Although use of gene-specific primers in RT markedly increased cDNA yield and, therefore, has the potential to significantly increase the sensitivity of the BCR-ABL1 test, the 19-base, gene-specific BCR-ABL1 RT primer caused high background noise in the electropherogram when a low level of BCR-ABL1 was loaded (Figure 4A). In an effort to identify and eliminate the source of this high background, PCR products associated with the background noise peaks were isolated and sequenced. Greater than 95% of the generated consensus sequences mapped to the *RANBP3* gene (Entrez Gene ID 8498) and its multiple splice isoforms, RANBP3-a, RANBP3-b, and RANBP-d. Based on this mapping, we identified significant homology between the 19-base, gene-specific BCR-ABL1 RT primer used and the RANBP3 transcript isoform family (Table 2). We hypothesized that these splice variants resulted in nonspecific electrophoretic peaks at lower input concentrations of BCR-ABL1 native product. To test this hypothesis, we lengthened the 19-base, gene-specific BCR-ABL1 RT primer by 10 bases at the 3′ end to minimize homology of the gene-specific BCR-ABL1 RT primer to the RANBP3 transcript isoform family.

In contrast to the 19-base RT primer, when low-level BCR-ABL1 was loaded into the RT reaction with 29-bp RT primer, the electrophoretic background noise was low (Figure 4A). Compared with yields obtained with RH, use of the extended BCR-ABL1 gene-specific RT primer and the original GUSB gene-specific RT primer increased BCR-ABL1 b3a2 yield by 8.5-fold ($P = 0.03$) and GUSB yield by 6.6-fold ($P = 0.007$), respectively, in RNA samples with low BCR-ABL1 levels. Because the yield of each gene was increased about the same amount, normalized (BCR-ABL1 b3a2 per 10^3 GUSB) values measured with RH versus gene-specific RT primers were not significantly different ($P = 0.17$) (Figure 4B).

Discussion

Accurate measurement of BCR-ABL1 load in peripheral blood samples is necessary for optimal management of

Table 2 Homology of BCR-ABL1 Gene-Specific RT Priming Site in ABL1 Transcript and RANBP3 Transcript Isoform Family

Transcript or primer	Sequence
ABL1 transcript	5′-CCTTCTCGCTGGACCCAGTGAAAATGACCCCAA-3′
BCR-ABL1 19-nucleotide gene-specific RT primer	5′-TTGGGGTCATTTCACTGG-3′
RANBP3 transcript isoform family	5′-GAGGAGAAAGAGCCCCAG∼AAAAATGAGTCCAG-3′
BCR-ABL1 29-nucleotide gene-specific RT primer	5′-TTGGGGTCATTTTCACTGGGTCCAGCGAGAAGGT-3′

The ∼ symbol represents a small insertion sequence (two to five bases) seen in a small fraction of captured splice variants (<1%). Complementary bases are boldfaced.

chronic myelogenous leukemia. The test and methods that we have developed should enable reliable interlaboratory comparison of results. The values obtained with the BCR-ABL1 kits have high correlation with results obtained in the CAP MRD-B−BCR-ABL1 p210 2011 Survey and from analysis of CML patient samples with an established commercial test in a clinical laboratory. The synthetic internal standards used in this kit were prepared in large volume and are stable, so that they may be shared with other laboratories. This approach enables reliable interlaboratory comparison and controls for intersample variation in interfering substances, interexperimental variation in quality or quantity of PCR reagents, and/or thermal cycler performance, all factors that may lead to incorrectly low measured values or false negatives. In addition, use of gene-specific primers in RT increased the sensitivity of the test by at least 10-fold to a maximum of 100-fold compared with RH primers, depending on the amount of RNA input in the RT reaction. This will enable reduction in amount of RNA required and/or measurement of MMR at a log-lower level.

To ensure reliable BCR-ABL1 measurement in clinical testing, it is clear that, in clinical application, optimal RT conditions, including primers and RNA input, should be established and adhered to, and that blood storage time before measurement should be kept to a minimum and kept relatively constant. The effects of varying these conditions are of sufficient magnitude to affect reliability of measuring a 0.5-$\log_{10}$ increase in BCR-ABL1 load, an accepted indication of treatment failure, and thereby could negatively affect patient treatment. We conclude that optimal measurement of BCR-ABL1 per 10^3 GUSB in whole blood is obtained when gene-specific primers are used in RT, and samples are processed within 24 hours after blood collection.

For monitoring CML, patient blood samples typically are collected at one site and then shipped to a specialized laboratory at a different site for BCR-ABL1 testing. Consequently, RNA may not be extracted from blood samples until >48 hours after collection. Our results, such as those of van der Velden[52] and Moravcova[53] and colleagues, suggest that blood samples kept at ambient temperature for extended amounts of time result in altered BCR-ABL1 measurement. Specifically, BCR-ABL1 b3a2 per 10^3 GUSB values decreased by approximately 50% in 48 hours (Figure 2). Such a sizable decrease, combined with other sources of analytical variation, could mask a 0.5-$\log_{10}$ increase. A delay of change in treatment as the result of inaccurate BCR-ABL1 measurement could lead to decreased survival time for patients. Although RIN score is commonly used as a measurement of RNA integrity, in this study, it was an insensitive indicator of RNA degradation. However, RIN score may be useful as a convenient first analysis.

To be effective, the BCR-ABL1 measurement method used for molecular CML monitoring must reliably measure low copy numbers. In quantitative RT-PCR−based assays that measure BCR-ABL1 load, the sensitivity of BCR-ABL1 detection can be heightened by increasing the amount of extracted RNA from patient samples into the RT reaction before PCR amplification. Because of increased analyte load, there is an advantage to loading up to 600 ng RNA/μL reaction (18 μg RNA/30 μL reaction) when RH are used for priming in the RT reactions. However, when RH are used, adding more RNA to the RT reaction only marginally improves sensitivity of BCR-ABL1 detection as the result of reduced RT cDNA yield (Figure 3B). Analysis with the RTSM confirmed that increasing RNA in the RT reaction inhibits RH-primed RT. Possible causes of decrease in RT efficiency in these conditions include limiting substrates, increased presence of blood-specific RT inhibitors, or a combination of both. In studies not presented, attempts to optimize substrate concentrations in RT did not improve RT efficiency with RH priming at a higher RNA load. In contrast, when gene-specific RT primers were used, BCR-ABL1 yield was markedly higher and there was no interference with RT as the level of RNA input into the RT reaction increased. When RNA input was increased from 0.9 to 18 μg RNA/30 μL RT reaction, there was a threefold increase in BCR-ABL1 yield with RH but a 158-fold increase with gene-specific primers, providing a nearly 50-fold higher yield of BCR-ABL1 product with gene-specific primers. Thus, if enough blood-derived RNA from a sample is available, increasing the RNA input of the RT reaction to as much as 600 ng RNA/μL reaction will markedly improve the sensitivity of BCR-ABL1 detection when gene-specific RT priming is used, but will provide only small improvement with RH primers.

Although oligo (d)T primers are commonly used for reverse transcription of eukaryotic mRNAs with a 3′ poly-A tail, they have reduced RT efficiency for BCR-ABL1 fusion transcript (Figure 3A), possibly because of the presence of a large distance (approximately 1000 nucleotides) between the poly-A tail and ABL1 exon 2.[24] As a result, BCR-ABL1 molecular monitoring laboratories generally use RH as the RT priming method instead of oligo (d)T primers.[30] Although the use of RH primers allows the reverse transcription of most transcripts from the same RNA sample, gene-specific RT priming is reported to provide more efficient RT of specific transcripts.[54] Previous studies showed that the use of random pentadecamer primers increased BCR-ABL1 yield by 86% compared with RH.[55] In our study, gene-specific RT primers produced the optimal yield of BCR-ABL1 b3a2 and GUSB cDNA by generally, 1 to 2 logs more than that obtained with RH, depending on the RNA input into the RT reaction.

In our construction of the extended BCR-ABL1 gene-specific RT primer to eliminate off-target RT priming, we determined that the RANBP3 transcript isoform Family has a significant degree of sequence homology to ABL1 in exon 2. This particular finding is important, because it may enable optimization of other nucleic acid−based CML monitoring methods to avoid false-positive measurement of RANBP3 transcripts.

We conclude that use of gene-specific RT primers will be optimal for molecular monitoring of BCR-ABL1 load and that this is especially important when BCR-ABL1 copy

number is low (eg, when patients achieve a major or complete molecular response). It is recognized that use of RH to prime blood-derived RNA for cDNA synthesis enables the reverse transcription of all possible BCR-ABL1 fusion transcripts, and this is optimal for initial CML diagnosis. However, because gene-specific primers have much higher RT efficiency, they are more suitable for monitoring purposes when the specific fusion transcript splice variant is known.

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