BCR-ABL1 minor breakpoint (e1a2) monitoring using an analytically validated multiplex assay

Michael Dodge, Ion Beldorth, Stela Filipovic-Sadic, Keri Masson, Marie Fahey, John Hedges, and Justin T Brown
Asuragen, Inc., Austin, TX

Summary

- This BCR-ABL1 e1a2-quantification assay improves workflow with its pre-mixed reagent formulation and multiplex assay format, and generates results sufficient for studies in deep molecular responses for minor breakpoint.
- The assay's limits were extensively characterized using background human RNA, matching the tissue of interest.
- Reproducible assay for minor breakpoint that accurately calls molecular responses of ≥4.5 logs of reduction.

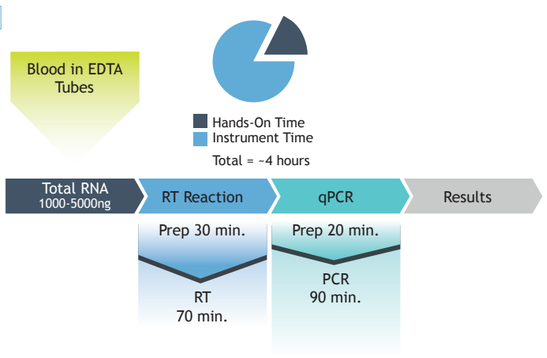
Introduction

Clinical research on molecular response in CML and B-ALL requires a highly optimized assay with well characterized analytical limits and will catalyze improved monitoring strategies. BCR-ABL1 e1a2 fusion transcript (minor breakpoint) of t(9;22) quantitation assesses tumor burden in Philadelphia-chromosome-positive precursor B-cell acute lymphoblastic leukemia (Ph+ B-ALL) and chronic myeloid leukemia (CML). Researchers require a reproducible assay for minor breakpoint that accurately calls molecular responses of ≥4 logs of reduction. However, interpretation of clinical research data sets can be confounded by use of different assays whose limits are not well characterized in the context of the tissue of interest (human leukocyte RNA). We describe the analytical validation and method comparison of a multiplex system reporting continuous BCR-ABL1:ABL1 %ratio values via automated analysis.

Methods

We developed reagents for RT-qPCR, with both steps performed on the ABI 7500 Fast Dx (ThermoFisher Scientific). Armored RNA Quant® (ARQ) molecules form a blend of nuclease-resistant BCR-ABL1 and ABL1 transcripts used to calibrate and control the system. Multiplexed 4-point curves using ARQ blends provide BCR-ABL1 and ABL1 copy values and account for the batch run-specific efficiency of the RT step. Controls (high, low, negative) were also developed. For most validation studies herein, cell-line RNA positive for e1a2 was diluted into non-leukemic leukocyte human RNA specimens to create challenge panels for precision, LOD, LOQ, and linearity studies. Cell-line RNAs were used to test specificity. Results from residual clinical specimen RNAs were compared to the BCR/ABL1 Quant Test (RUO). Software was also developed that automatically analyzes raw SDS files for %ratio values and contains a logic algorithm that flags any specimen requiring further review.

1A



1B

% BCR-ABL1:ABL1	LR
100	0.0
32	0.5
10	1.0
3.2	1.5
1	2.0
0.32	2.5
0.1	3.0
0.032	3.5
0.01	4.0
0.0032	4.5
0.0025	4.6
0.001	5.0

Figure 1. Assay workflow and analytical values. A) The minor breakpoint assay has a simple workflow. Whole blood in EDTA is obtained and leukocyte-enriched total RNA is prepared. Using 1000-5000 ng in the RT (from 100-500 ng/µL) facilitates accurate measurement of BCR-ABL1 and ABL1. Total hands-on-time is ~1 hour and on-board instrument time was ≤4 hours. **B)** The system yields the percentage ratio of BCR-ABL1 to the control gene ABL1. These values are expressed in a linear space and are more normally distributed after logarithmic transformation (for example, see Q-Q plot in Figure 3). The Log Reduction (LR) values used in the analytical validation studies herein are the $\log_{10}$ reduction from theoretical totality, or 100%. Therefore, LR = 2 – $\log_{10}$(%ratio). After performing the appropriate statistical analysis, a %ratio value was conversely determined as %ratio = $10^{(2\text{-}LR)}$. The table provides a summary of several LR values and their corresponding %ratio values for reference. (We note that the international scale values of %IS are well established for the Major breakpoints e13a2 and e14a2, but that such a scale has not been determined for the minor breakpoint.)

Conflict of Interest Disclosure. All authors have the financial relationship to disclose: Employment by Asuragen.

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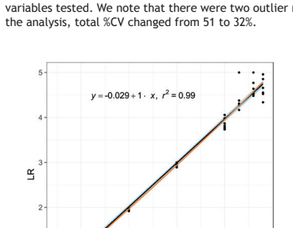
Results

Specimen ID for human RNA	Positive	Step 1: Exclude ≤1 copy/qPCR BCR-ABL1	Step 2: Further exclude ≤0.0010%
PE10	12/30 (40%)	5/30 (17%)	5/30 (17%)
PE15	5/30 (17%)	0/30 (0%)	0/30 (0%)
PE20	4/30 (13%)	0/30 (0%)	0/30 (0%)
PE21	3/30 (10%)	1/30 (3%)	1/30 (3%)
All 4 specimens	24/120 (20%)	6/120 (5%)	6/120 (5%)

Figure 2. Limit of Blank (LOB) study. We tested 4 unique non-leukemic human RNA specimens presumed to be negative for BCR-ABL1. Testing was adapted from CLSI EP17-A2 and spanned 2 lots, 2 operators, 3 runs, 2 calendar days, and 2 qPCR instruments at 1000 ng/RT. Out of 120 valid measurements, 96 results were "Undetected (Sufficient ABL1)" and 24 were positive for BCR-ABL1. The LOB was determined by classical non-parametric ranking at the 95%ile as nearly **1 copy/qPCR BCR-ABL1**. That is, specimens that show ≤1 copy/qPCR BCR-ABL1 may be indistinguishable from non-leukemic specimens. LOB for %ratio by classical non-parametric ranking at the 95%ile was **0.0010% (LR5.00)** (data not shown). **Table:** We assessed the level of false positivity after application of the LOB in 2 steps. Excluding specimens ≤1 copy/qPCR BCR-ABL1 generated no %ratio values ≤0.0010%. Overall false positivity decreased from 20% to 5.0%.



Specimen ID	Count	%Pos	Mean(LR)	SD(LR)*	Pass/Fail§	Mean(%r)†
PE22	30	100%	4.303	0.249	pass	0.0050
PE23	30	100%	4.436	0.211	pass	0.0037
PE24	29	97%	4.638	0.370	fail	0.0023
PE25	30	100%	4.447	0.253	pass	0.0036

*Performed in Excel via function STDEV.S()
§Against precision criteria of SD ≤0.29 for LR3.5-4.25, or SD ≤0.36 for >LR4.25.
†Calculated as %ratio = $10^{(2\text{-}LR)}$. Specifically, the mean of LR values was first determined, and then antilog-converted to determine a geometric mean of %ratio values.

Figure 3. Limit of Detection (LOD) and Limit of Quantitation (LOQ) studies. Multiple methods were used. **Determination of LOD in a human RNA background:** SUP-B15 e1a2 positive cell Line RNA was diluted into four unique human CML-negative RNA samples and then tested at 1000 ng/RT based upon methods adapted from CLSI EP17-A2, spanning 2 lots, 3 batch runs, 2 days, 3 operators, and 2 instruments. (One panel member contained an analyte level below the anticipated LOD and was therefore excluded from LOD analysis.) The log-transformed LR values were normally distributed (Shapiro-Wilk test, n=90, p-value = 0.24) **(Q-Q plot at left).** The classical parametric method generated a corrected overall SD of LR values of 0.238 with a 95%ile of 0.393. Therefore, LOD = LR5.00(LOB) – 0.393 = LR4.61 – 0.0025%. **Informational assessment of LOD in a cell line RNA background:** SUP-B15 RNA was diluted into HL-60 RNA. Using the same method described above, we obtained an estimated LOD of 0.0005% (LR5.31) across 80 valid measurements (with 66 detectable %ratio values). Further, one specimen was 95% positive (19/20) at a mean of 0.0008% (LR5.11). **Determination of LOQ in a human RNA background:** The LOQ was estimated using the same specimens used in the LOD assessment above. The lowest analyte level that passed the acceptance criteria (SD≤0.36 at LR4.25 or greater) measured LR4.45, which supports an LOQ of 0.0036%. **Informational assessment of LOQ in a cell line RNA background:** Using the same method described above, we obtained an estimated LOQ of 0.0002% (LR5.70) across 80 valid measurements. The LOD and LOQ observations indicate that the use of cell line background RNA for sensitivity studies is not as challenging as the matrix of interest, human RNA.

Target %ratio	Mean %ratio	Lot %CV	Operator %CV	Instrument %CV	Day/Run %CV	Within Run %CV	Residual %CV	Total %CV
10	10.5	0.0	0.0	8.3	0.0	0.0	34.5	42.8
1	1.11	6.6	0.0	1.5	0.0	7.6	11.9	27.6
0.1	0.11	13.9	0.0	0.0	0.0	3.9	33.7	51.4
0.01	0.01	0.0	0.0	2.6	0.0	0.0	38.5	41.1

Target LR	Mean LR	Lot SD	Operator SD	Instrument SD	Day/Run SD	Within Run SD	Residual SD	Total SD
1	0.98	0.00	0.00	0.03	0.00	0.00	0.10	0.12
2	1.95	0.05	0.00	0.00	0.00	0.01	0.10	0.17
3	2.96	0.03	0.00	0.01	0.00	0.03	0.05	0.12
4	3.98	0.00	0.00	0.00	0.00	0.00	0.17	0.17

Figure 4. Single-site Precision. SUP-B15 e1a2 positive cell Line RNA was diluted into human CML-negative RNA and tested at 1000ng/RT. Testing was adapted from CLSI EP05-A3 and spanned 3 lots, 2 operators, 8 runs, 8 days, and 2 qPCR instruments, generating 192 valid measurements. The observed variability is displayed in the tables above, one expressed in %ratio and the other in LR values. Overall, measurements were reproducible within all variables tested. We note that there were two outlier replicates in the data set, both for the specimen targeted to 0.1%. When these were removed from the analysis, total %CV changed from 51 to 32%.

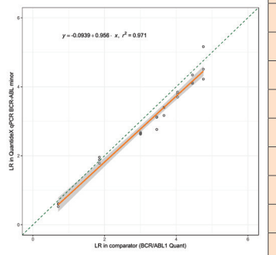
Figure 5. Linearity study. SUP-B15 e1a2 positive cell line RNA was diluted into human CML-negative RNA. Testing was based upon CLSI EP6-A, spanning 2 lots and 2 batch runs and generating 57 valid measurements from 1000 ng/RT. Across both lots, measurements showed a linear regression curve with slope of 1.01, intercept of -0.03, and R2 of 0.99. Measured samples ranged from LR0.61 (25%) to LR4.65 (0.0022%), with a maximum SD of 0.30. Additionally, all second and third order coefficients were statistically insignificant with (p > 0.05), indicating that the test was linear across the range. Therefore, the method is **linear from at least 0.0025% to 25%** (LR4.61 to LR0.61), with the lower limit constrained by LOD. Observed LR values are plotted against the targeted LR values for each sample. Replicates are shown (n=6 per targeted sample). The gray line represents the 1st order regression line (intercept -0.0286, slope 1.013, R2 0.9898), with the 2nd order line shown in orange. The 95%CI is shaded in blue.

Cell Line Specimen Type	Fusion Transcript	Result
CML	t(9;22) Major breakpoint e13a2 (p210)	>LOD (see below)
CML	t(9;22) Major breakpoint e14a2 (p210)	>LOD (see below)
AML	t(8;21) AML1/ETO	<LOD (6/6 Undetected)
AML M3 / APL	t(15;17) PML/RARA	<LOD (5/6 Undetected)
AML M4	inv(16) CBFB/MYH11	<LOD (5/6 Undetected)
AML M5	t(9;11) MLLT3/MLL	<LOD (6/6 Undetected)
ALL	t(12;21) TEL/AML1	<LOD (6/6 Undetected)
ALL	t(1;19) E2A/PBX1	<LOD (5/6 Undetected)
ALL	t(4;11) MLL/AF4 (e10e4)	<LOD (6/6 Undetected)

Residual Clinical Specimen Breakpoint	Result in QuantideX® qPCR BCR-ABL IS Kit (Major)	Result in QuantideX® qPCR BCR-ABL minor Kit	Fold Difference*
e13a2	87%IS (MR0.06)	0.0331% (LR3.48)	2,600x
e13a2	6.8%IS (MR1.17)	<LOQ at 0.0029% (LR4.54)	2,300x
e13a2	2.3%IS (MR1.64)	<LOD at 0.0011% (LR4.94)	2,100x
e14a2	62%IS (MR0.21)	<LOD at 0.0017% (LR4.76)	36,000x
e14a2	13%IS (MR0.89)	Undetected (5/6) and <LOB at 0.0005% (LR5.15) (1/6)	N/A and 26,000x

*Estimated as a ratio of specific signal (Major breakpoint in IS Kit) to non-specific signal (Major breakpoint in minor Kit)

Figure 6. Analytical Specificity (Exclusivity). Exclusivity was assessed by testing 9 leukemic specimens positive for CML, AML, or ALL at 1000 ng/RT, across 2 lots of Kit, 2 operators, and 2 days, genreating 54 valid measurements. As seen in the table on the left, undiluted cell line RNA specimens known to express a very high level of the Major breakpoints e13a2 and e14a2 generated positive signal for e1a2 at low levels in this assay. The primer sites in e1 and a2 exons exist within e13a2 and e14a2 RNA; however, the reaction is predicted to be inefficient due to the larger amplicon and therefore cross-detects at a low level. To determine the level at which Major-breakpoint-positive specimens become undetectable in the minor breakpoint assay, multiple dilutions of e13a2-positive and e14a2-positive residual clinical human RNA specimens into human CML-negative RNA were tested in parallel between Asuragen's BCR-ABL1 assays and the results are displayed in the table at right.



Clinical Specimen Accession ID	BCR/ABL1 Quant Test (Major & minor)	QuantideX® qPCR BCR-ABL minor Kit	QuantideX® qPCR BCR-ABL IS Kit (Major)	Interpretation
PE75	0.0091% (LR4.04)	0.0166% (LR3.78)	Undetected (sufficient ABL1)	minor only
PE76	2.1038% (LR1.68)	3/3 were <LOD at 0.0011% (LR4.96)	3.1434%IS (MR1.50)	predominantly Major
PE77	Undetected (sufficient ABL1)	Undetected (sufficient ABL1)	Undetected (sufficient ABL1)	undetected
PE78	15.7903% (LR0.80)	0.0266% (LR3.58)	30.8752%IS (MR0.51)	predominantly Major
PE79	19.6958% (LR0.71)	26.3027% (LR0.58)	Undetected (sufficient ABL1)	minor only
PE80	Undetected (sufficient ABL1)	Undetected (sufficient ABL1)	Undetected (sufficient ABL1)	undetected
PE81	Undetected (sufficient ABL1)	Undetected (sufficient ABL1)	Undetected (sufficient ABL1)	undetected
PE82	Undetected (sufficient ABL1)	2/3 were <LOD at 0.0005% (LR5.30)	Undetected (sufficient ABL1)	undetected
PE83	0.0997% (LR3.00)	0.2228% (LR2.65)	Undetected (sufficient ABL1)	minor only
PE84	Undetected (sufficient ABL1)	3/3 were <LOD at 0.0016% (LR4.79)	Undetected (sufficient ABL1)	undetected (possible high neg)
PE85	0.0350% (LR3.46)	0.1083% (LR3.00)	Fail (low ABL1)	minor only (low ABL1)
PE86	1.3907% (LR1.86)	1.3615% (LR1.87)	Undetected (sufficient ABL1)	minor only
PE87	0.0035% (LR4.45)	0.0067% (LR4.18)	Undetected (sufficient ABL1)	minor only
PE88	Undetected (sufficient ABL1)	1/3 was <LOD at 0.0004% (LR5.44)	Undetected (sufficient ABL1)	undetected
PE89	0.0018% (LR4.76)	0.0032% (LR4.63)	Undetected (sufficient ABL1)	minor only
PE90	0.0220% (LR3.66)	0.0491% (LR3.32)	Undetected (sufficient ABL1)	minor only
PE91	>LOQ at 60.1958% (LR0.22)	0.0299% (LR3.53)	>LOQ at 88.3307% (MR0.05)	predominantly Major

Figure 7. Method Comparison. De-identified blood specimens were acquired through a 6-site collection protocol with IRB approvals for any individual with e1a2 confirmed previously by an unnamed, independent method. Specimens were blinded for Major breakpoint status. All specimens were analyzed at 1,000 ng/RT in both the Test (across 3 lots, singleton per lot) and a unique reagent set as a comparator (1 lot in singleton of BCR/AB1 Quant). The Test quantifies minor breakpoint (e1a2), and the comparator reagent set quantifies Major and minor breakpoints without distinguishing between them. Three specimens were not correlated. Discrepancy analysis demonstrated that they were highly positive in a test specific for Major breakpoints. The remaining 14 specimens were fully concordant (6 <LOD in both assays, 8 measurable in both assays), for an overall agreement of 100%. **Correlation plot (left).** Samples that were positive in both assays (and negative for Major breakpoint) are charted. A dotted, dark green unity line is shown for reference. Six undetected (<LOD) data sets are not shown. The 8 measurable e1a2 specimens were highly correlated for LR values (slope near 1, y-intercept near 0, Pearson R of 98.5%). Bias appeared minimal and uniform. **Table of measurable and qualitative results across multiple tests (right).** Where replicates were performed across 3 lots (minor kit), the mean is shown for %IS and geometric mean is shown for LR value.

Conclusions

- The QuantideX® qPCR BCR-ABL minor assay showed sensitive, multiplex detection of e1a2 and ABL1 with results returned as copy number and BCR-ABL1:ABL1 %ratio values.
- Limits were determined in a background of human RNA specimens: LOB = 1 copy/qPCR and 0.0010%. LOD = 0.0025%. LOQ = 0.0036%.
- Linearity was observed from at least 0.0025 to 25%.
- Single-site precision was acceptable at all levels through the dynamic range.
- A method comparison demonstrated good concordance and correlation with a previously developed assay.

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