Validation of a Complete Solution for BCR-ABL1 Monitoring of Both Major and Minor Breakpoints that Reports ABL1 Copy Number

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Summary

- The QuantideX® qPCR BCR-ABL Portfolio improves workflow with streamlined reagent formulation and multiplex assay format, and generate results sufficient for studies in deep molecular responses for Major and minor breakpoints.
- The assays' limits were extensively characterized using background human RNA matching the tissue of interest.
- Robust and reproducible assays for Major and minor breakpoints accurately assess molecular responses of ≥4.5 logs of reduction.

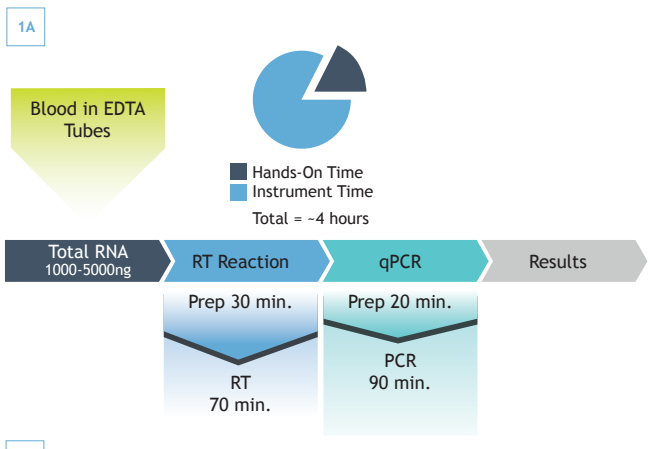
Introduction

Quantitation of BCR-ABL1 e13a2 and/or e14a2 (Major breakpoint, M-BCR) and e1a2 (minor breakpoint, m-BCR) fusion transcripts of t(9;22) assesses tumor burden in CML. As the extent of the efficacy of TKI therapy has been demonstrated over the past 2 decades, it has become evident that deep analytical sensitivity is required to assess response and clearance. Further, interpretation of clinical and 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 of a multiplex system for Major breakpoint providing continuous ABL1 copy and MR values, analytical sensitivity >MR4.5, and direct traceability to the WHO Primary BCR-ABL1 materials. We also describe analytical validation of a multiplex system for minor breakpoint reporting continuous ABL1 copy and %ratio values with analytical sensitivity of >4.5 logs. These studies were performed using human RNA background materials.

Methods

We developed reagents for RT-qPCR for Major and minor breakpoints. 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 Major breakpoints, a single 4-point curve using ARQ blends mimics the WHO Primary BCR-ABL1 reference materials. A lot-specific $2^{-\Delta Ct}$ calculation provides ABL1 copies/qPCR for evaluation of endogenous control levels. For minor breakpoint, two multiplexed 4-point curves using ARQ technology provide BCR-ABL1 and ABL1 copy values. For most validation studies herein, either human RNA positive for a Major breakpoint or cell-line RNA positive for minor breakpoint was diluted into non-leukemic leukocyte human RNA specimens to create challenge panels. Software was also developed that automatically analyzes raw SDS files for ratios and contains a logic algorithm that flags any specimen requiring further review. Validation studies were performed on the ABI 7500 Fast Dx for both RT and qPCR steps.

1A



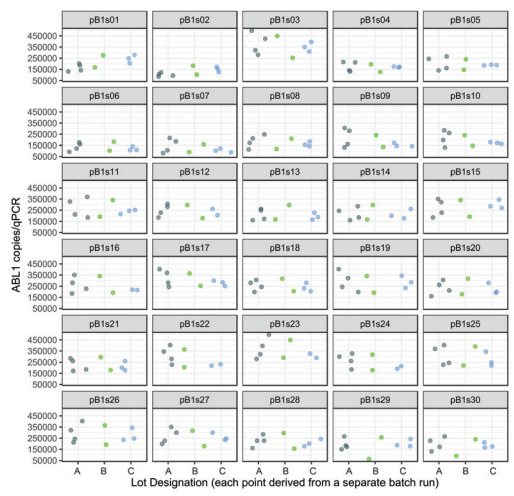
1B

% BCR-ABL1: ABL1 or %IS	LR or MR
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
0.002	4.7
0.001	5.0

Figure 1. Assay workflow and analytical values. Panel A: The Major and minor breakpoint assays have the same, 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 was ~1 hour and on-board instrument time was ≤4 hours. **Panel 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 Molecular Response (MR for Major) and Log Reduction (LR for minor) values used in the analytical validation studies herein are the log10 reduction from theoretical totality, or 100%. Therefore, LR = 2 - log10(%ratio) and MR = 2 - log10(%IS). After performing the appropriate statistical analysis, a linear value was conversely determined as %ratio = 10^(2 - LR) and %IS = 10%(2 - MR). The table provides a summary of several MR / LR values and their corresponding linear 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.)

Results

2A

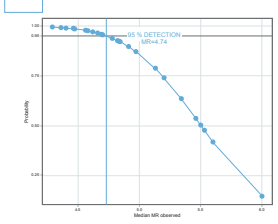


2B

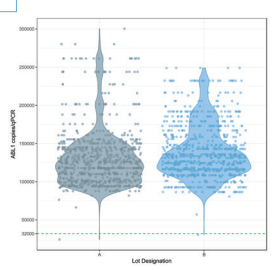
Specimen ID for human RNA	Positive in QuantideX® qPCR BCR-ABL minor Kit	Step 1: Exclude ≤1 copy/qPCR BCR-ABL1	Step 2: Further exclude ≤0.0010%
PE10	12/30 (40%)	5/30 (17%)	5/30 (17%)
PE15	5/30 (17%)	0/30 (0%)	0/30 (0%)
PE20	4/30 (13%)	0/30 (0%)	0/30 (0%)
PE21	3/30 (10%)	1/30 (3%)	1/30 (3%)
All 4 specimens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

Figure 2. Limit of Blank (LOB) studies. Testing was adapted from CLSI EP17-A2. **Major breakpoint LOB** was estimated by testing 30 separate, non-leukemic human RNA specimens. Testing spanned 3 lots, 4 operators, 9 runs, 4 calendar days, and 4 qPCR instruments, yielding 270 possible test results. Five (5) tests resulted in "Fail (baseline error)". "Undetected (Sufficient ABL1)" was reported for 265 test results and 2 results were reported "Below LOD" at MR5.71 (0.0002%IS) and MR5.35 (0.0004%IS). This yielded an LOB at the 95%ile of **"Undetected (Sufficient ABL1)" for Major** (95% CI of 97.0-99.9%). The two positive results were reported as "Undetected" when repeated in two subsequent runs each. **Panel A: ABL1 copies observed in the LOB study.** Within-lot and between-lot variability across all ABL1 measurements was assessed visually. Individual points are shown in a jitter plot with transparency. **Minor breakpoint LOB:** We tested 4 unique non-leukemic human RNA specimens presumed to be negative for BCR-ABL1. Testing spanned 2 lots, 2 operators, 3 runs, 2 calendar days, and 2 qPCR instruments. 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 e1a2.** 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). **Panel B:** We assessed the level of unexpected positivity after application of the LOB in 2 steps. Excluding specimens ≤1 copy/qPCR BCR-ABL1 generated no %ratio values ≤0.0010%. Overall unexpected positivity decreased from 20% to 5.0%—all 6 of which ranged from 1.1 to 2.4 copies/qPCR.

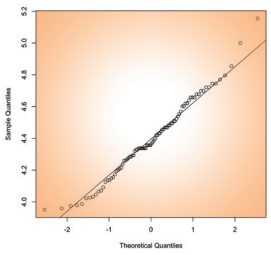
3A



3B



3C



3D

Specimen ID (e1a2)	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-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. Testing was adapted from CLSI EP17-A2. **Panel A: LOD for Major in human RNA.** Four (4) separate BCR-ABL1 positive human RNA specimens (2 of e13a2, 2 of e14a2) were diluted into 4 different human CML-negative RNA specimens to create a panel. Each of the 28 dilution levels was tested a total of 60 times at 1000 ng/RT. Testing spanned 2 lots, 4 operators, 40 runs, 15 calendar days, and 4 qPCR instruments. This yielded 1680 possible measurements. The fitted Probit model is shown. This analysis estimates 95% positivity at MR4.74 (95% CI ±0.03) corresponding to 0.0018%IS. The positivity estimate of **MR4.7** was maintained for each lot and for each of the two transcripts (e13a2 and e14a2) and via the non-parametric method described in CLSI EP17-A2 (while controlling the type II error at 5%). The %CV for samples at or near the LOD range was 73%. LOQ was determined to be MR4.87 (0.0016%IS) (data not shown); therefore, LOQ was constrained by LOD as MR4.7. **Panel B: ABL1 copies observed in the LOD study.** Within-lot and between-lot variability across all 1680 ABL1 measurements was assessed visually using a Stingray plot. Individual points are shown in a jitter plot with transparency and then overlaid with violin plots of the points' distributions. **Note** that there is biological and process variability contributing to the distributions; specifically, identical ABL1 values are not expected between the 4 background RNAs (biological) or 28 resultant RNA blends (process). **LOD for minor 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, 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) and visually by Q-Q plot in **Panel C.** 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, Panel D:** 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 LOD 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.

4A

%IS (Major)			MR (Major)		
Target	Mean	%CV	Target	Mean	SD
10%IS	18.1906	13.7	1	0.697	0.092
1%IS	2.0787	15.8	2	1.634	0.069
0.1%IS	0.2031	20.9	3	2.658	0.053
0.032%IS	0.0628	29.4	3.5	3.185	0.077
0.01%IS	0.0197	29.9	4	3.675	0.092

4B

%ratio (minor)			LR (minor)		
Target	Mean	%CV	Target	Mean	SD
10%	10.5	42.8	1	0.98	0.12
1%	1.11	27.6	2	1.95	0.17
0.1%	0.11	51.4	3	2.96	0.12
0.01%	0.010	41.1	4	3.98	0.17

Figure 4. Precision Studies. Testing was adapted from CLSI EP05-A3. **Panel A: Multi-site Precision for Major.** Five dilution series were constructed with five samples in each pool (at each dilution level) for a total of 25 samples. Each sample was evaluated at 3 sites by 2 operators making quintuplicate measurements on 2 days for a total of 750 measurements. Overall, the determination of MR values was reproducible within all variables tested (the maximum observed standard deviation was 0.099 for a single specimen at the MR4 target level). This supports the testing of specimens in singleton in the test. **Panel B: Single-site Precision for minor.** 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%.

5A

Cell Line Specimen Type	Fusion Transcript	Result in QuantideX® qPCR BCR-ABL minor Kit
CML	t(9;22) Major breakpoint e13a2 (p210)	>LOD (see below)
CML	t(9;22) Major breakpoint e14a2 (p210)	>LOD (see below)
AML M2	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)

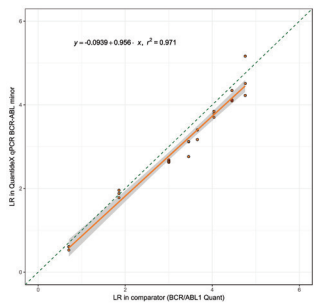
5B

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 5. Analytical Specificity (Exclusivity) for QuantideX® qPCR BCR-ABL minor Kit. 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, generating 54 valid measurements. As seen in the table in **Panel A**, 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 in **Panel B.**

6A



6B

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 6. Method Comparison for QuantideX® qPCR BCR-ABL minor Kit. 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 CE IVD). The Test quantifies minor breakpoint (e1a2), and the comparator reagent set quantifies Major and minor breakpoints without distinguishing between them. **Concordance assessment (Panel B).** Fourteen (14) specimens were correlated. Discrepancy analysis on the 3 non-correlated specimens 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 (Panel A). 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 (Panel B)**. 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 IS Kit and QuantideX® qPCR BCR-ABL minor Kit showed sensitive, multiplex detection of Major and minor breakpoints with ABL1 copy number and BCR-ABL1:ABL1 values.
- Limits were determined in a background of human RNA specimens
  - » Major, LOB = Undetected. LOD = 0.0020% (MR4.7). LOQ = 0.0020% (MR4.7).
  - » minor, LOB = 1 copy/qPCR and 0.0010% (LR5.0). LOD = 0.0025% (LR4.6). LOQ = 0.0036% (LR4.4).
- Precision was acceptable at all levels through the dynamic range.
- A method comparison for minor breakpoint demonstrated concordance and correlation with a previously developed, CE-marked IVD kit.

*Presenting

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

Major and minor breakpoint assays are CE marked for IVD use.

This project has been funded in part with Federal funds from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services, under Contract No. HHSN261201500009C.

Presented at ESH iCMLf John Goldman Conference 2017 (Estoril, Portugal)

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