Expanding Access to the QuantideX® qPCR BCR-ABL IS Monitoring Assays Through Deployment on Multiple Quantitative Real-Time Instruments

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Summary

- The QuantideX® qPCR BCR-ABL IS monitoring assay on the **cobas® z** 480 Analyzer (Roche) generates precise results with high sensitivity sufficient for studies in deep molecular responses (>MR4.5).
- The assay reports ABL1 copy numbers, MR values, and %IS values traceable to the WHO primary reference materials, with single-click sample QC and analysis using QuantideX® Reporter software.
- The assay was characterized extensively using human RNA, which demonstrates the true functionality of the assay in clinical§ applications.

Introduction

The monitoring of BCR-ABL1 e13a2 and e14a2 (Major breakpoint, M-BCR) fusion transcript levels with reverse transcription and quantitative polymerase chain reaction (RT-qPCR) is the established standard of care for assessing tumor burden reduction in Chronic Myeloid Leukemia (CML) treated with tyrosine kinase inhibitors (TKIs). Recent advancements in research investigating TKI treatment efficacy and therapy discontinuation have led to the establishment of new guidelines for BCR-ABL1 assays, recommending analytical sensitivity sufficient to detect deep molecular responses (MR) of at least 4.5 logs below the standardized International Scale (IS) baseline. Thus, a critical need exists for validated BCR-ABL1 assays standardized to the IS and able to accurately detect at or above MR4.5. While we have previously analytically and clinically validated the QuantideX® qPCR BCR-ABL IS assay on the ABI 7500 Fast Dx (Thermo Fisher), we set out to validate this assay on the **cobas® z** 480 Analyzer (Roche) to maximize availability. Here, we show data from the analytical validation of the QuantideX® qPCR BCR-ABL IS assay. Additionally, we present data from the initial characterization of the QuantideX® qPCR BCR-ABL minor assay used to monitor e1a2 breakpoints on the **cobas® z** 480 Analyzer, bringing a complete solution to this widely accessible diagnostic platform.

Methods

The Major and minor breakpoint monitoring assays use Armored RNA Quant® (ARQ) technology to deliver a highly reproducible nuclease-resistant standard curve containing both BCR-ABL1 and ABL1 transcripts. The calibrators are traceable to the WHO Primary BCR-ABL1 reference materials for the Major breakpoint assay. Both assays contain three controls (high, low, and negative) that maintain high accuracy and precision from lot to lot. For all Major breakpoint studies, human RNAs positive for e13a2 or e14a2 were diluted into non-leukemic human RNA to create challenge panels for studies such as precision, LOD, LOQ, and linearity. Software was also developed that automatically analyzes exported files from the **cobas® z** 480 UDF software (v2.0) for %IS and MR values and contains a logic algorithm that flags any specimen requiring further review.

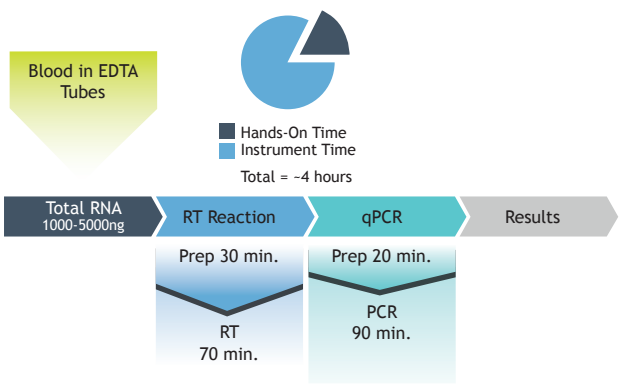


Figure 1. Assay workflow. The Major and minor monitoring assays both have the same simple workflow. Whole blood in EDTA is obtained and leukocyte-enriched total RNA prepared. Using 1000-5000 ng in the RT (from 100-500 ng/µL) facilitates accurate measurement of BCR-ABL1 and ABL1. Total hands-on-time is ~1 hour and on-board instrument time was ≤4 hours.

Results

	% Detected	Median %IS	MR*
Overall	97%	0.0020	4.7
e13a2 (both lots)	96%	0.0023	4.7
e14a2 (both lots)	98%	0.0019	4.7
Lot 1 (both breakpoints)	95%	0.0019	4.7
Lot 2 (both breakpoints)	98%	0.0022	4.7

*MR values were obtained from Median %IS rounded to the thousandths based on assay precision

Figure 2. Limit of Detection (Major breakpoints). CML-positive human RNA with either e13a2 or e14a2 fusions was diluted into a background of CML-negative human RNA to a targeted MR of 4.7 to create 7 unique specimens (3 e13a2, 4 e14a2), each tested at the minimum RNA input of the assay (1000ng/RT). Testing consisted of 2 reagent lots, 6 batch runs, 3 days, 1 operator, and 2 instruments, generating a total of 210 measurements. Data was analyzed by classical nonparametric analysis (CLSI EP17-2A) using %IS values. Final values were rounded to the thousandths place based on assay precision and converted to MR values. The LOD of the assay was nearly indistinguishable between breakpoints and lots, and was consistent with the previously validated BCR-ABL1 monitoring assay on the 7500 Fast Dx platform. Limit of Blank was also analyzed using 9 CML-negative samples with two reagent lots and eight batch runs, generating a total of 142 measurements. Testing yielded a results of Undetected (sufficient ABL1) overall and for both lots.

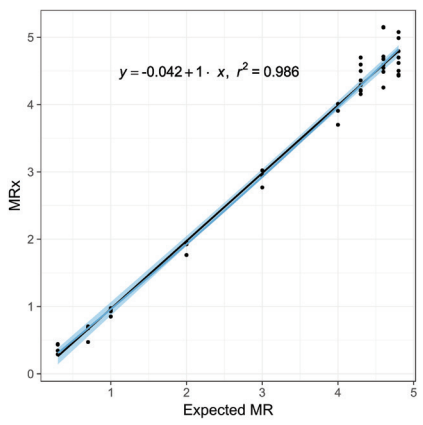
Sample	Breakpoint	%Detected	Mean MR	SD	%IS
mmxD1s01	e13a2	100%	4.53	0.30	0.0032
mmxD1s02r	e13a2	97%	4.74	0.29	0.0020
mmxD1s03	e13a2	90%	4.64	0.25	0.0025
mmxD1s05	**e14a2**	**97%**	**4.81**	**0.23**	**0.0017**
mmxD1s06	e14a2	97%	4.77	0.24	0.0019
mmxD1s07	e14a2	100%	4.55	0.28	0.0031
mmxD1s08	e14a2	97%	4.78	0.28	0.0018

Figure 3. Limit of Quantitation (Major breakpoints). The same dataset used to determine LOD was used to estimate the LOQ of the assay. The study generated 210 measurements across 7 samples and 2 reagent lots. Data from both lots were pooled for analysis. The standard deviations of all 7 samples were below pre-defined criteria (SD≤0.36 for MR>4.25). As per CLSI guidelines (EP17-A2), the sample with the lowest reproducible measurement (highest MR) is taken as the LOQ (MR4.81). However, the LOQ of the assay is constrained by the LOD of the assay; therefore, LOQ=LOD=MR4.7.

Target MR	Breakpoint	Instrument SD	Lot SD	Operator SD	Run/ Day SD	Within SD	Total SD
1	e13a2	0.01	0.00	0.01	0.04	0.03	0.10
1	e14a2	0.01	0.00	0.00	0.04	0.05	0.10
2	e13a2	0.00	0.03	0.02	0.02	0.05	0.12
2	e14a2	0.00	0.01	0.03	0.03	0.04	0.12
3	e13a2	0.01	0.02	0.00	0.06	0.04	0.13
3	e14a2	0.00	0.03	0.03	0.06	0.04	0.16
4	e13a2	0.03	0.05	0.00	0.09	0.07	0.23
4	e14a2	0.00	0.00	0.03	0.07	0.08	0.18

Figure 4. Single-Site Precision (Major breakpoints). CML-positive human RNA with either e13a2 or e14a2 fusions was diluted into a background of CML-negative human RNA to four targeted MR values spanning the range of the assay to create 8 unique specimens (4 e13a2, 4 e14a2), each tested at 1000ng/RT. Testing took place over 24 runs using 2 instruments, 3 reagent lots, and 2 operators, with 2 runs on different days for each instrument-lot-operator combination, a total of 384 measurements. Run-to-run variability was therefore confounded with day-to-day variability. Data was analyzed by ANOVA, producing standard deviations broken down by error source. Instrument, lot, and operator variability were all low, with 84% of the total SD coming from a combination of run, day, and within-run variability on average across all 8 samples. Overall standard deviations for all samples were also low, and were within pre-defined error criteria (SD≤0.21 for MR<3.5, SD≤0.29 for 3.5≤MR≤4.25, SD ≤0.36 for MR>4.25). This breakdown is ideal, as the assay is very reproducible between instruments, lots, and operators.

e13a2 Correlation Plot



e14a2 Correlation Plot

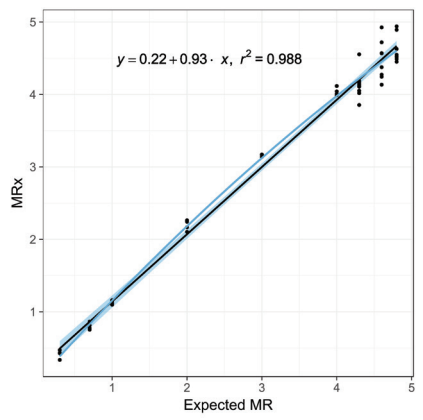


Figure 5. Linearity (Major breakpoints). CML-positive human RNA with either e13a2 or e14a2 fusions was diluted into a background of CML-negative human RNA to nine targeted to MR values from MR0.3 to 4.8 to create 18 unique specimens (9 per breakpoint), each tested at 1000ng/RT. Testing took place over two runs using two reagent lots. Data was analyzed for each breakpoint individually according to CLSI EP06-A, where 1st, 2nd, and 3rd order least squares regression models are fitted to the dataset. All 18 samples had standard deviations within error criteria (SD≤0.21 for MR<3.5, SD≤0.29 for 3.5≤MR≤4.25, SD ≤0.36 for MR>4.25). Both breakpoints had statistically significant linear models (black lines, equations shown). The e14a2 breakpoint also had a statistically significant 2nd order coefficient when modelled by 2nd order regression (blue line), but was within error of the linear model at every targeted MR, indicating an acceptably low degree of nonlinearity. Thus, the assay was linear from MR0.30 to MR4.80 for both breakpoints.

e1a2 Monitoring Assay z 480 (preliminary testing)	% Ratio (e1a2/ABL1)
LOB	0.0010
LOD	0.0023
LOQ	0.0023
Linear Range	20 to 0.0018

Figure 6. Preliminary characterization of the minor breakpoint assay on the z 480. We have also completed preliminary testing of a minor breakpoint assay (e1a2) with similar design to the Major breakpoint assay on the **cobas® z** 480 instrument. CML-positive SUP-B15 cell line RNA containing e1a2 was diluted into a background of CML-negative human RNA for testing. Methods, study designs, and numbers of measurements were similar to those presented herein for the Major breakpoint monitoring assay. Performance characteristics for this assay were also similar to previous 7500 Fast Dx testing.

Conclusions

- Deep characterization of the assay on the **cobas® z** 480 using human RNA produced equivalent or superior performance characteristics as compared to the validated QuantideX® qPCR BCR-ABL IS Kit on the 7500 Fast Dx.
  - » LOD/LOQ = MR4.70 (0.0020%IS)
  - » Linear from MR0.30 to MR4.80 (50 to 0.0016%IS) for e13a2 and e14a2
  - » High degree of reproducibility between lots, operators, instruments, and samples
- Preliminary characterization of the BCR-ABL minor (e1a2) assay on the z 480 suggests comparable portability to the **cobas® z** 480.

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

§Research use only. Not yet CE marked on the **cobas® z** 480.

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