BCR-ABL1 MONITORING ON THE IS USING A CLINICALLY AND ANALYTICALLY VALIDATED MULTIPLEX ASSAY DIRECTLY ALIGNED TO THE WHO PRIMARY STANDARDS THAT UNIFIES REPORTING FORMATS

Ion Beldorth, Walairat Laosinchai-Wolf, Marie Fahey, John Hedges, Bernard Andruss, and Justin T Brown
Asuragen, Inc., Austin, TX

SUMMARY

- The QuantideX® qPCR BCR-ABL IS Kit showed sensitive, multiplex detection of e13a2, e14a2, and *ABL1* on the ABI 7500 Fast Dx with direct reporting on the International Scale (IS) and as Molecular Reduction (MR) Values.
- Limits: LOB was "Undetected". LOD and LOQ were both MR4.7 (0.002%IS).
- Linearity was observed from at least MR0.3 (50%IS) to MR4.7 (0.002%IS).
- Multi-site precision (reproducibility) was verified as a maximum SD of 0.9 at MR3.7 (30%CV at 0.02%IS).
- RNA mass input was validated from 1000-5000 ng/RT (MR1.0-MR4.7).
- A multi-center clinical outcome study confirmed the statistically significant difference in EFS at 32-40 months stratified by achievement of MR3 using the Test run at 12-18 months after start of TKI.

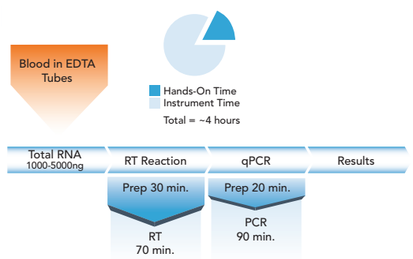
INTRODUCTION

Chronic Myeloid Leukemia is a disorder that results when a translocation between chromosome 9 and chromosome 22 lead to an active fusion gene *BCR-ABL1*. Detection of *BCR-ABL1* e13a2 or e14a2 fusion transcripts (major breakpoint, M-BCR) of t(9;22) is important in studying tumor burden in CML. To facilitate this, the International Scale (IS) was established to standardize the reporting of these transcripts relative to a common baseline. As newer TKI therapies create deeper responses with lower numbers of circulating leukemic cells, analytical sensitivity has become a critical topic in investigations into TKI discontinuation, where researchers require an assay that confidently calls molecular responses of ≥4.5 logs below baseline (0.0032%IS or MR4.5). This has led to various reporting formats as a patient achieves deep response over time, creating a non-contiguous language of monitoring including: baseline, 10%IS, 1%IS, MMR, MR4, and MR4.5. We describe the analytical and clinical validation of a multiplex assay system reporting continuous MR values via automated software analysis, clinical accuracy at MR3, analytical sensitivity of MR4.7, and direct traceability to the WHO Primary *BCR-ABL1* reference materials without requiring establishment and revalidation of a conversion factor.

METHODS

We developed reagents for the QuantideX qPCR BCR-ABL IS Kit, both steps performed on the ABI 7500 Fast Dx. Armored RNA Quant® (ARQ) technology was employed to generate a blend of nuclease-resistant *BCR-ABL1* and *ABL1* RNA transcripts to calibrate and control the system. A single four-point standard curve using ARQ blends mimics the WHO Primary *BCR-ABL1* reference materials and accounts for the relative batch run-specific efficiency of the RT step. cDNA generation and qPCR were optimized, including allowance of high mass of nucleic acid without inhibition. Software was developed, including a floating, traceable logic algorithm to ensure that sufficient *ABL1* was detected to protect this LOD. A multi-center clinical outcome study was conducted at 3 clinical laboratories to validate clinical monitoring.

1A



1B

Response designation	MR	%IS
Baseline	0.0	100
	0.5	32
PCyR	1.0	10
	1.5	3.2
CCyR	2.0	1
	2.5	0.32
MMR	3.0	0.1
	3.5	0.032
	4.0	0.01
CMR	4.5	0.0032
	4.7	0.002
	5.0	0.001

Figure 1. Assay workflow and reportable values. (A) The *BCR-ABL1* assay is designed to have a simple workflow. Whole blood in EDTA is obtained and a leukocyte-enriched RNA at 1000-5000 ng ensures accurate measurement of *BCR-ABL1* and *ABL1* targets. Total RNA is subjected to a reverse transcription reaction to yield cDNA for subsequent qPCR analysis. Total hands-on-time is estimated at 1 hour and total on board instrument time was validated as ≤4 hours. **(B)** The numerical value of the WHO International Scale is %IS, the ratio, expressed as a percentage, of *BCR-ABL1* expression to the expression of a control gene (ABL1 for the Test). The MR value is the log10 reduction from the internationally standardized baseline (100%IS). The Test reports MR values and %IS. At its inception, the IS was reported by a local laboratory by determining a ratio of *BCR-ABL1* in arbitrary linear units (e.g. number of analyte molecules) to *ABL1* in the same units, multiplied by 100%, and then multiplied by a laboratory-specific correction factor. Such %IS values are now traceable to the WHO primary reference materials. The IS is most normally distributed after logarithmic transformation. MR values represent such a transformation. The table provides a summary of several MR values (MR = 2 – log10(%IS)) and their corresponding %IS values (%IS= 10^(2-MR)) for reference.

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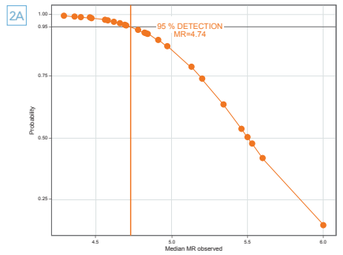
RESULTS

2A



2B

LOD Determination					
Transcript	Total Results	Total Positive	Percent Undetected	LOD (%IS)	LOD (MR)
e13a2	179	172	3.9	0.002	4.70
e14a2	420	400	4.8	0.002	4.70

Figure 2. Limit of Blank (LOB) and Limit of Detection (LOD) studies. The LOB was estimated by testing 30 separate, non-leukemic human RNA specimens that were presumed to be negative for *BCR-ABL1* (data not shown). The specimens ranged from 1000-5000 ng/RT. Testing was compliant with CLSI EP17-A2 and 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 95th percentile of "Undetected (Sufficient *ABL1*)" (95% CI of 97.0-99.9%). The two positive results were reported as "Undetected" when repeated in two subsequent runs each. **(A)** We characterized the performance of the test at very low *BCR-ABL1* levels using a Probit approach. Four separate *BCR-ABL1* positive human RNA specimens (2 of e13a2, 2 of e14a2) were diluted in different human CML-negative RNA specimens to create a panel. Each of the 28 dilution levels was tested a total of 60 times at the minimum 1000 ng input. 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). **(B)** This region was then used to assess LOD via the non-parametric method described in CLSI EP17-A2, while controlling the type II error at 5%. The median value of the tested values across all included panel member replicates was determined and defined as the LOD. This analysis yielded an LOD for each transcript of 0.002%IS / MR4.7, consistent with the prediction of the Probit approach. The %CV for the LOD range was 73.

Sample	Predominant Breakpoint	All measurements					
		Mean(MR)	SD(MR)	Mean(%IS)	%CV(%IS)	Undetected	Detected
pC1s03	e13a2	4.79	0.27	0.0020	70.2	5	15
pC1s10	e14a2	4.67	0.23	0.0025	56.1	1	19
pC1s11	e14a2	4.80	0.34	0.0021	90.7	3	16
pC1s18	e14a2	4.60	0.24	0.0029	58.5	0	20
pC1s19	e14a2	4.87	0.25	0.0016	65.6	5	15
pD1s03	e13a2	4.82	0.25	0.0018	62.2	1	19

Figure 3. Limit of Quantitation (LOQ) study. The LOQ was estimated by testing 4 separate human RNA specimens that were positive for *BCR-ABL1*, each diluted into RNAs from human CML-negative whole blood specimens to a target of MR4.7. The panel members were tested at the system's minimum of 1000 ng/RT. Testing was compliant with CLSI EP17-A2 and generated 120 measurements. Across two lots of Kit, the highest MR value was 4.87 (0.0016%IS), and the highest standard deviation (SD) was 0.27. Additionally, we reviewed the LOD study, whose panel had 25 members with measured MR values ≥4.5, all yielding SD values ≤0.33. Overall, no SD was greater than the specification of 0.36, supporting an LOQ of at least MR4.7 (0.002%IS).

Targeted MR	Sample	Site	Day	Operator	Within	Total
		SD	SD	SD	SD	SD
MR1	pA1s01	0.008	0.000	0.012	0.028	0.049
	pA1s06	0.009	0.014	0.000	0.021	0.044
	pA1s11	0.024	0.017	0.014	0.022	0.077
	pA1s16	0.017	0.020	0.000	0.019	0.056
	pA1s21	0.013	0.021	0.011	0.020	0.064
MR2	pA1s02	0.000	0.021	0.000	0.068	0.089
	pA1s07	0.000	0.022	0.004	0.017	0.044
	pA1s12	0.032	0.009	0.026	0.021	0.087
	pA1s17	0.012	0.016	0.009	0.019	0.055
	pA1s22	0.012	0.014	0.000	0.031	0.057
MR3	pA1s03	0.039	0.024	0.005	0.036	0.104
	pA1s08	0.000	0.023	0.009	0.028	0.059
	pA1s13	0.039	0.026	0.000	0.052	0.117
	pA1s18	0.018	0.000	0.009	0.033	0.059
	pA1s23	0.036	0.019	0.000	0.032	0.087
MR3.5	pA1s04	0.061	0.023	0.026	0.037	0.147
	pA1s09	0.013	0.042	0.010	0.043	0.108
	pA1s14	0.038	0.039	0.041	0.051	0.169
	pA1s19	0.026	0.010	0.000	0.035	0.071
	pA1s24	0.050	0.027	0.000	0.032	0.109
MR4	pA1s05	0.054	0.027	0.032	0.053	0.167
	pA1s10	0.029	0.000	0.000	0.067	0.095
	pA1s15	0.014	0.037	0.000	0.070	0.122
	pA1s20	0.042	0.000	0.000	0.079	0.121
	pA1s25	0.026	0.028	0.005	0.063	0.123

Figure 4. Multi-site precision (reproducibility) for all samples and pools in the four-site study. Five pools (dilution series) with target MR values of 1, 2, 3, 3.5, and 4 were constructed with five samples in each pool (at each dilution level), for a total of 25 samples. Each sample was evaluated at four sites by at least two operators on at least two days for a total of 1200 measurements. This dataset was analyzed using a nested random effect analysis of variance using the REML criterion in R version 3.2.5. and SAS PROC MIXED v9.3. The components estimated were: Site to site, Day to day within operator and site, Operator to operator within site, and Within run. The variance components for the samples grouped by pool are summarized. For each pool and sample, the SD for each source of variance is shown. Overall, the determination of MR values was reproducible within all variables tested (the maximum observed standard deviation was 0.169). This supports the testing of specimens in singleton.

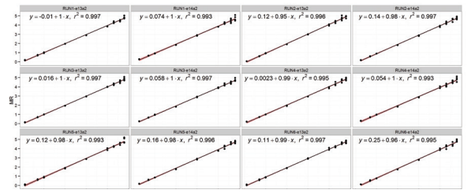


Figure 5. Linearity study. Linearity was estimated by testing 2 separate human RNA specimens that were positive for *BCR-ABL1*, one expressing predominantly e13a2 and one e14a2. Each was diluted into RNAs from human CML-negative whole blood specimens to target ranges of MR0.1 to 4.8. The panel members were tested at 3000 ng/RT. Testing was compliant with CLSI EP06-A and generated 144 measurements. Across two lots of Kit, the two transcripts showed indistinguishable linear regression curves as demonstrated by identical slopes of 1.01 and intercepts of -0.11 and -0.05 for e13a2 and e14a2, respectively. The standard deviations (SD) of the test specimens at each level were statistically equal to or less than the criteria established for the study. Breakpoint e13a2 measured MR0.12 to MR4.84, with a maximum SD of 0.17. Breakpoint e14a2 measured MR0.22 to MR4.78, also with a maximum SD of 0.17. Additionally, 2nd- and 3rd-order polynomial regression fits were assessed. From the 2nd-order fit, the absolute deviations from 1st-order linearity were all ≤0.08 MR units across the entire range. This supports an observation of linearity from at least MR0.3 (50%IS) to MR4.7 (0.002%IS). Above are shown individual plots of observed MR values versus expected MR values. Overall, the slope of each curve is between 0.95 and 1.00, with r2 values greater than 0.993 and intercepts of 0.03 and 0.18, indicating strong linearity for each transcript within each run. Confidence intervals for the linear fit are shown in red.

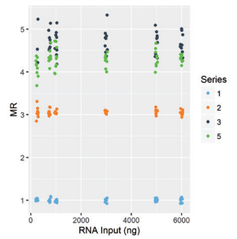


Figure 6. RNA Mass Input Assessment. RNA input was evaluated using a panel of human *BCR-ABL1* positive RNA specimens diluted into human CML-negative RNA specimens, creating levels ranging from 250ng to 6000ng RNA and spanning MR values from MR1.0 to MR4.7. Nine replicates were tested at each input amount. For MR1.0 (Series 1), MR3.0 (Series 2), and MR4.3 (Series 5) samples, the reproducibility was high across the range of inputs tested. Precision was also high at all input levels at MR4.7 (Series 3), with the exception of the 250ng input level. All 9 replicates were detected at all MR values and RNA mass input levels except for the lowest mass inputs at MR4.7 (equivalent to LOD)—1000ng (8/9), 750ng (8/9), 250ng (3/9). These results support the recommended input range of 1000 to 5000 ng.

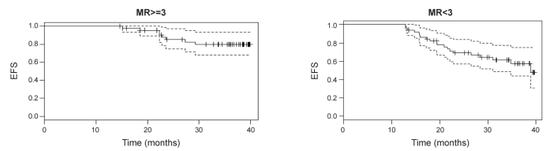


Figure 7. Clinical performance. A multi-center clinical outcome study was conducted at 3 US clinical laboratory sites to assess clinical monitoring using the Test's measurements. A total of 139 samples from 98 patients were collected and enrolled in the clinical study at 2 clinical sites. A total of 137 evaluable samples were present from 96 of those subjects. Clinical outcome evaluation was performed for determination of event-free survival (EFS) at 32-40 months among chronic phase CML patients having the Test run at 12-18 months after being on any TKI medication. Primary endpoint was assessed by the probability of at least one event by the endpoint 32-40 months after initiation of TKI treatment as estimated from the Kaplan-Meier survival function. The null hypothesis was the event probabilities of the group attaining MR3.0 and of the group not attaining MR3.0 are equal. The alternative was that the 36-month event-free probability of the group attaining MR3.0 is greater than those not attaining MR3.0. The log-rank test statistic for no difference between the groups was 6.05, P=0.028, confirming the statistically significant difference. The two survival curves are shown with Greenwood 95%CI.

CONCLUSIONS

- The QuantideX qPCR BCR-ABL IS Kit improves workflow with its streamlined reagent formulation and multiplex assay format, facilitates assessment on the IS without conversion (through integrated ARQ materials traceable to the WHO Primary), reports results on a continuous scale (as both MR and %IS values), and has sensitivity sufficient to assess deep molecular responses.
- It has also been clinically validated for stratification by MR3 in a multi-center clinical outcome study.

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