# Development and evaluation of a quantitative multiplex assay for the harmonization of BCR-ABL1 measurement on the international reporting scale

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## SUMMARY

- The BCR/ABL1 Quant™ assay is a research tool designed and manufactured under cGMP to ensure product quality and lot-to-lot consistency.*
- The reagents showed sensitive, multiplex detection of e1a2, b2a2, b3a2, and ABL1.
- The assay has the preliminary performance** required for quantitative measurement of BCR-ABL1 expression compatible with reporting on the International Scale.
- High qualitative and quantitative correlation with existing simplex laboratory-developed tests was observed.

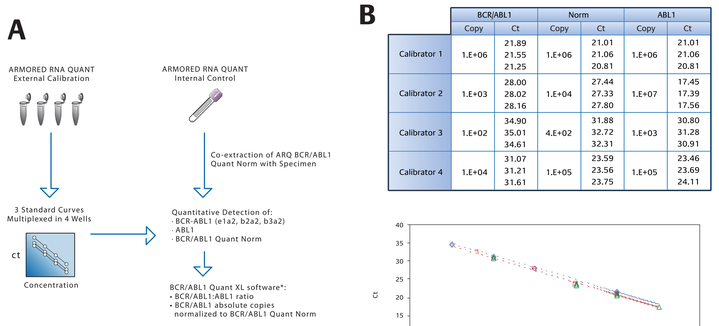
## INTRODUCTION

The IRIS trial and follow-up studies have demonstrated the correlation between established treatment response criteria in chronic myeloid leukemia (CML) patients and the quantity of BCR-ABL1 fusion transcripts. Sensitive molecular methods to quantify the levels of BCR-ABL1 fusion transcripts have therefore emerged as valuable tools for the assessment of treatment response and detection of relapse. However, the use of different platforms, assay designs, internal control genes, standard curve materials, and reporting methods by clinical laboratories worldwide has resulted in the inconsistent interpretation of individual patient response and comparison of response rates between patients. The International Scale (IS) was created to enable laboratories to express their results as BCR-ABL1 IS levels corresponding to specific molecular and clinical landmarks.

We previously reported the development and evaluation of the BCR/ABL1 Quant™ (RUO*) assay, a research tool for simultaneous amplification and detection of three BCR-ABL1 fusion transcripts (e1a2, b2a2, b3a2), ABL1 (an endogenous internal control), and BCR/ABL1 Quant™ Norm (an exogenous internal control). The assay includes reverse transcription (RT), multiplex real-time PCR with TaqMan® probes, quantification of each target relative to Armored RNA Quant® standard curves, and the optional determination of fusion transcript identity (e1a2, b2a2, or b3a2) via size fractionation by capillary electrophoresis (CE). Here we show how strict cGMP processes and quality controls enable maintenance of assay performance for quantitative measurement of BCR-ABL1 to ABL1 ratio relative to an IS Reference Method and to simplex laboratory-developed tests (LDT) routinely used in testing laboratories.

## MATERIALS & METHODS

All BCR/ABL1 Quant™ assays were performed according to the instructions for use (Asuragen Inc.) on a real-time PCR instrument from the 7500 series (Applied Biosystems). Residual specimens were collected at 2 independent sites, each using their own pre-analytical and analytical methods to collect, process, and test the samples. All residual human specimens in this study were de-identified and evaluated according to protocols approved by their respective institutions. For analytical experiments, total RNA was purified from translocation-positive leukemic cell lines (SUP-B15/e1a2, BV-173/b2a2, or K562/b3a2) or from the t(9;22)-negative leukemic cell line HL-60. Positive cell line RNA was diluted mass-to-mass in a background of negative cell line RNA keeping the total RNA input constant at 1,500 ng per RT reaction. Establishment and validation of a conversion factor (CF) was performed as described in Branford et al. (Blood, 2008), except that cell line RNAs were used as specimens. For capillary analysis, the PCR reactions were diluted 1 in 50 in water, heat denatured in formamide and analyzed on a 3130xl instrument (Applied Biosystems) equipped with 36 cm POP-7 capillaries. Nucleotide (nt) sizes were determined using the GeneScan™ 500 ROX™ Size Standard and the GeneMapper® Software V4.0.



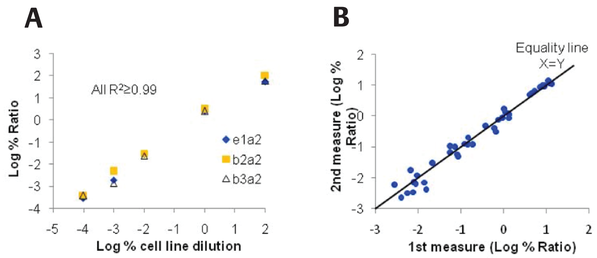

B

| | BCR/ABL1 | | Norm | | ABL1 | |
|---|---|---|---|---|---|---|
| | Copy | Ct | Copy | Ct | Copy | Ct |
| Calibrator 1 | 1.E+06 | 21.89<br>21.55<br>21.25 | 1.E+06 | 21.01<br>21.06<br>20.81 | 1.E+06 | 21.01<br>21.06<br>20.81 |
| Calibrator 2 | 1.E+03 | 28.00<br>28.02<br>28.16 | 1.E+04 | 27.44<br>27.33<br>27.80 | 1.E+07 | 17.45<br>17.39<br>17.56 |
| Calibrator 3 | 1.E+02 | 34.90<br>35.01<br>34.61 | 4.E+02 | 31.88<br>32.72<br>32.31 | 1.E+03 | 30.80<br>31.28<br>30.91 |
| Calibrator 4 | 1.E+04 | 31.07<br>31.21<br>31.61 | 1.E+05 | 23.59<br>23.56<br>23.75 | 1.E+05 | 23.46<br>23.69<br>24.11 |




**Figure 1.** (A) Assay Calibration and Control with Armored RNA® Quant™ (ARQ) Technology and Sample Processing Workflow. The copy number of each target and the ratio of BCR/ABL1 to ABL1 copy number are calculated based on 3 calibration curves generated. The external Calibrator Set consists of 4 vials only, each containing a blend of precisely quantified BCR/ABL1, ABL1, and BCR/ABL1 Quant™ Norm ARQs mixed at different concentrations. In addition, a known fixed copy number of BCR/ABL1 Quant™ Norm ARQ can be spiked into the specimen lysate prior to or during RNA isolation (exogenous internal process control) for monitoring of process efficiency and absolute quantification of BCR/ABL1 copy number (for example, normalized copy number of BCR/ABL1 per mL of blood). (B) Representative examples of calibration curves. Following heat denaturation, the 4 ARQ calibrators were run in triplicate to evaluate repeatability. The resulting Ct (left) were automatically processed through the software to build 3 standard curves for BCR/ABL1, ABL1 and Norm (right) covering 5 Logs of copies per RT. R2 were all >0.99.

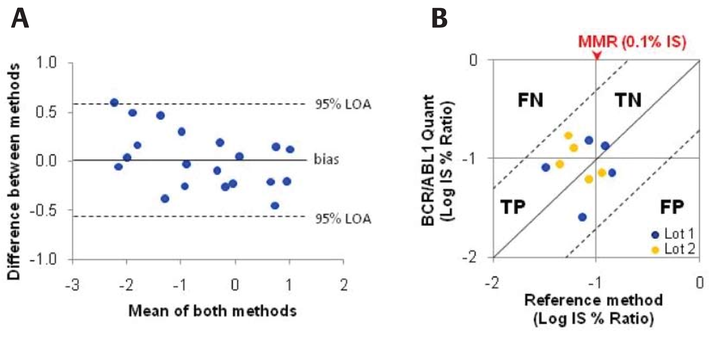
## RESULTS




C

| | | Run 1 User 1 | Run 2 User 1 | Run 3 User 1 | Run 4 User 2 | Run 5 User 3 | Mean | % CV |
|---|---|---|---|---|---|---|---|---|
| BCR-ABL1 | Slope | -3.41 | -3.58 | -3.33 | -3.35 | -3.22 | -3.38 | 3.9 |
| | Intercept (Ct) | 41.89 | 42.89 | 41.22 | 41.86 | 40.87 | 41.75 | 1.9 |
| | R square | 0.994 | 0.995 | 0.991 | 0.995 | 0.994 | 0.994 | 0.2 |
| ABL1 | Slope | -3.52 | -3.41 | -3.41 | -3.56 | -3.5 | -3.48 | 1.9 |
| | Intercept (Ct) | 41.1 | 41.05 | 41.06 | 41.84 | 40.61 | 41.13 | 1.1 |
| | R square | 0.998 | 0.998 | 0.999 | 0.999 | 0.999 | 0.999 | 0.1 |
| Log % Ratio | 1:100 dilution | 0.75 | 0.76 | 0.86 | 0.68 | 0.62 | 0.73 | 12.1 |
| | 1:100,000 dilution | -2.22 | -2.03 | -2.14 | -2.85 | -2.54 | -2.36 | 14.2 |

**Figure 2. Analytical Characterization.** Analytical performance assessed using synthetic in vitro transcript RNA showed a LOQ of 10-15 copies of BCR-ABL1 per PCR and a LOD of 2-5 copies per PCR with a linear dynamic range greater than 6 logs (data not shown). **(A)** Representative examples of analytical sensitivity and linearity with 3 leukemic cell line RNAs diluted in a background of HL-60 total RNA. The graphs show the mean values obtained from duplicate testing (undiluted or diluted 1 in 102 or 1 in 104), triplicate testing (diluted 1 in 105) or quadruplicate testing (diluted 1 in 106). **(B)** Results from duplicate testing using 40 independent cell line RNA dilutions at 1,500 ng input. The graph shows the percent ratio for the second measure plotted against the percent ratio of the first measure and the theoretical equality line (first measure = second measure). The calculated 95% limit of agreement on this set was plus or minus 2.4-fold indicating that singleton testing results were not significantly different from the mean of duplicate testing. **(C)** Evaluation of reproducibility for standard curves and BCR-ABL1 to ABL1 ratio across 5 independent runs performed by 3 different operators.



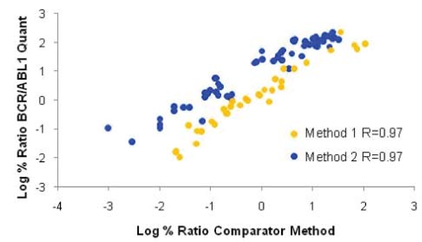

C

| | Establishment (lot 1) | Validation (lot2) | Overall |
|---|---|---|---|
| Number of samples tested | 20 | 20 | 40 |
| MMR negative by IS method | 12 (60%) | 11 (55%) | 23 (57.5%) |
| MMR positive by IS method | 8 (40%) | 9 (45%) | 17 (42.5%) |
| Agreement with IS method | 18 (90%) | 17 (85%) | 35 (87.5%) |
| Paired correlation | 0.97 | 0.98 | 0.97 |
| Mean bias | 0.017 | -0.019 | -0.004 |
| Conversion Factor | 1.04 | 0.96 | 0.99 |
| 95% limit of agreement | 3.6-fold | 4.2-fold | 3.9-fold |

**Figure 3: Comparison to the international scale.** **(A)** Bland and Altman analysis for the 20 samples tested with BCR/ABL1 Quant™ lot 1 at Asuragen and by the reference method at an IS Reference Laboratory (SA Pathology, Adelaide). The graph shows the difference between methods (log IS percent ratio minus log BCR/ABL1 Quant™ percent ratio) plotted against the average of both log percent ratio values. The mean difference between methods (bias, solid line) and the 95% limit of agreement between methods (95% LOA, dash lines) are shown. The conversion factor (CF) is the antilog of the mean bias (0.017). Fourteen months later, a validation experiment was performed using an independent lot of BCR/ABL1 Quant™ reagents. The bias after conversion was close to 0 and the antilog of the bias (CF) was close to 1 (see Figure 3B), indicating that there were no changes between reagent lots and methods. **(B)** Summary of results and assay performance during establishment and validation of the CF and for the combined data (overall). **(C)** Correlation plot between the IS percent ratios obtained with the BCR/ABL1 Quant™ assay and the reference method for samples within 3-fold to the MMR point (0.1% IS) according to the reference method. The equality line (solid line) and the 5-fold limit of agreement between methods (dash lines) are shown. The difference in IS % ratio measured by both methods for the 5 misclassified samples was indistinguishable from the inherent within-assay variability of each method (95% limit of agreement of plus or minus 2.5-fold). TN=true MMR negative. TP=true MMR positive. FN=false MMR negative. FP=false MMR positive.

A

| | |
|---|---|
| Number of total RNA samples tested | 115 |
| % samples from bone marrow specimens | 30% |
| % samples from peripheral blood specimens | 70% |
| % samples with minor BCR-ABL1 (e1a2) | 10% |
| % samples with major BCR-ABL1 (b2a2/b3a2) | 90% |
| Samples with quantitative BCR/ABL1 Quant data | 115 |
| Samples with quantitative LDT data | 103 |
| Paired correlation between methods | 0.97 |
| % agreement between methods (major/minor) | 100% |



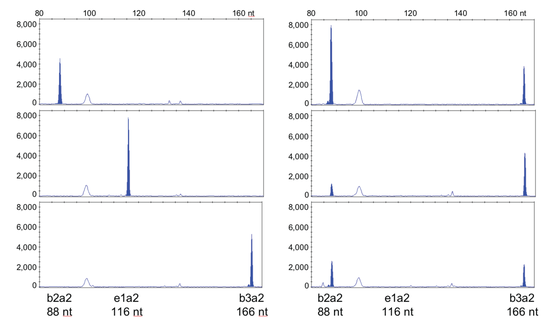




**Figure 4. Comparison to existing laboratory-developed tests.** **(A)** Description of sample set and summary of results. Specimens processed at HUP (method 1, n=33) were tested with the BCR/ABL1 Quant™ assay and a standard 7500 instrument at HUP. Specimens acquired from an independent clinical laboratory (method 2, n=82) were tested at Asuragen on a 7500 Fast Dx. Each specimen had previously been tested with independent simplex LDT specific of each site. The percent agreement between methods was calculated by comparing the qualitative results obtained by CE analysis with the BCR/ABL1 Quant™ amplicons versus the quantitative results obtained with independent LDTs specific either for the major (b2a2 and b3a2) or minor (e1a2) BCR-ABL1 fusion transcripts. **(B)** Quantitative analysis on 103 archived total RNA samples with LDT results ranging from greater than 100% ratio to about 0.001% ratio (12 samples had no quantitative LDT data and were reported as positive but below LOD of LDT). Percent ratio and calculated paired Pearson correlation values are shown for each independent method. (C) Representative examples of capillary electrophoresis traces for BCR-ABL1 amplicons (FAM channel).

## CONCLUSIONS

The BCR/ABL1 Quant™ RUO* assay showed high qualitative and quantitative agreement with independent simplex LDT and an IS reference method. The use of streamlined and optimized RT-qPCR reagents manufactured under cGMP combined with precisely quantified Armored RNA Quant calibrators anchored to a NIST standard enables the accurate and reproducible measure of BCR-ABL1 to ABL1 ratio**. Further multi-site clinical validation of these research reagents would likely facilitate harmonization of BCR-ABL1 quantitative measurement, improve laboratories' efficiency and workflow, and increase adherence to the current recommendations for reporting on the IS.

Conflict of interest disclosure: All authors are employees of Asuragen, Inc.

### Acknowledgments

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*For research use only. Not for use in diagnostic procedures.
**Preliminary research data. The performance characteristics of this assay have not yet been established.