Evaluation of BCR/ABL1 Quant Assay (RUO)* for the Quantitative Detection of e1a2, b2a2, and b3a2 Fusion Transcripts

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SUMMARY

- BCR/ABL1 Quant is a multiplex assay with a streamlined workflow for the detection of ABL1 and BCR/ABL1 fusion transcripts.
- Multiplexing and compatibility with capillary electrophoresis enables the detection and identification of 3 BCR/ABL1 fusion transcripts: b2a2, b3a2 and e1a2.
- Inclusion of Armored RNA® Quant™ Norm enables internal assay calibration for absolute quantification of BCR/ABL1 copy number.
- Assay validation at GenPath (BRL) increased the laboratory throughput and improved results reporting by including e1a2 data, percent ratio, and normalized copy number.

INTRODUCTION

The Philadelphia translocation, t(9;22), which juxtaposes the ABL1 proto-oncogene to the BCR gene generating a chimeric BCR/ABL1 gene, is the hallmark of chronic myeloid leukemia (CML). Fusion transcripts corresponding to BCR/ABL1 b2a2 or b3a2 are present in >95% of CML patients. About 5% of children with acute lymphoblastic leukemia (ALL) and 20-35% of adult ALL also carry t(9;22), most often e1a2. This molecular signature provides a tool for initial diagnosis, risk-stratified patient management, and monitoring of residual disease and potential recurrence during targeted therapy with imatinib mesylate.

Asuragen's BCR/ABL1 Quant (RUO)* is a research tool for simultaneous amplification and detection of three BCR/ABL1 fusion transcripts (e1a2, b2a2, b3a2), ABL1 (an endogenous control), and BCR/ABL1Quant Norm (an exogenous control) using total RNA extracted from human blood, bone marrow, or cultured cells. The assay uses multiplex reverse transcription-PCR (RT-PCR) in combination with real-time TaqMan® technology. The resulting PCR products are compatible with capillary electrophoresis (CE) for subsequent determination of the fusion transcripts identity (e1a2, b2a2, or b3a2) via size fractionation. Previous studies showed that the assay has the required analytical sensitivity and linear dynamic range to detect >5 Log decrease in b2a2 or b3a2 to ABL1 ratio. The objective of this study was to further evaluate the performance of the assay for the quantification of e1a2 and the reporting of absolute copy number of BCR/ABL1 fusion transcripts.

MATERIALS & METHODS

Peripheral blood or bone marrow from leukemia patients were collected as part of standard clinical care and following a protocol approved by Bio-Reference Laboratories. Total RNA was isolated from white blood cell enrichment pellets using the QIAamp RNA Blood Mini Kit (QIAGEN) according to the manufacturer's instructions. Asuragen's BCR/ABL1 Quant (RUO) kit was evaluated using 5 uL of archived total RNA following the Package Insert (500-2,000 ng RNA input recommended). Briefly, RNA was reverse transcribed into cDNA and then amplified by multiplex PCR using target-specific primers (37°C for 15 min, 95°C for 10 min followed by 45 cycles of 95°C for 15 sec and 60°C for 1 min) and the ABI 7500 Real-Time PCR System. Target copy number and BCR/ABL1:ABL1 ratio were calculated from 4 point standard curves generated with the provided ARQ calibrators. This was achieved by processing the raw data files from the ABI 7500 instrument using the BCR/ABL1 Quant XL analysis tool (Asuragen) in conjunction with Microsoft Excel. For CE analysis, PCR products were diluted 1:100 and 1 uL was mixed with 1 uL of ROX ladder and 13 uL of HiDi™ Formamide (Applied Biosystems). After heat denaturation at 95 C for 2 min, samples were transferred on a cold block, quickly centrifuged, and analyzed on an ABI 3130 Genetic Analyzer using a 50 cm POP-7 capillary (Pre Run: 15 kV for 180 sec; Temperature: 60°C; Injection: 1.6 kV for 20 sec; Run: 15 kV for 50 min; Other settings = default).

RESULTS

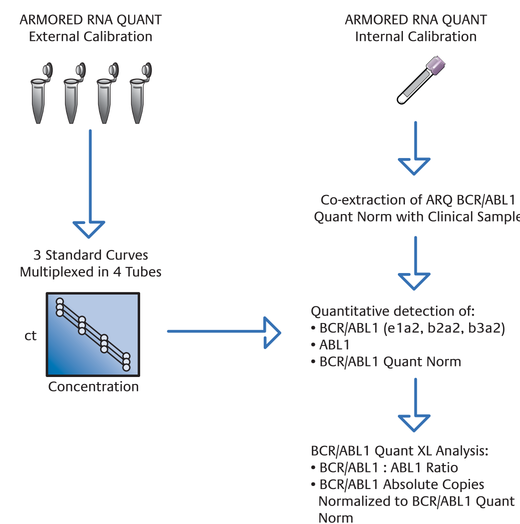


Figure 1. Assay Calibration with Armored RNA® Quant™ (ARQ) Technology and Sample Processing Workflow. The copy number of each target and the ratio of BCR/ABL1 to ABL1 copy number are calculated based on 3 calibration curves generated with each run. The external Calibrator Set consists of 4 vials only, 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).

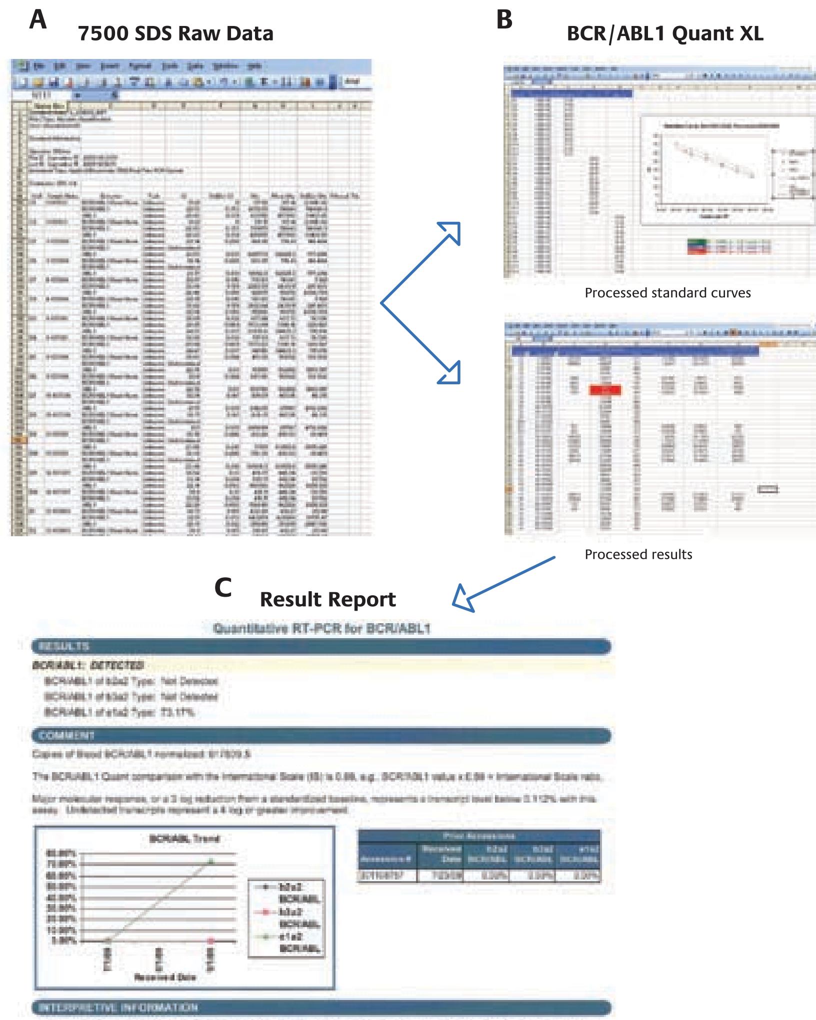
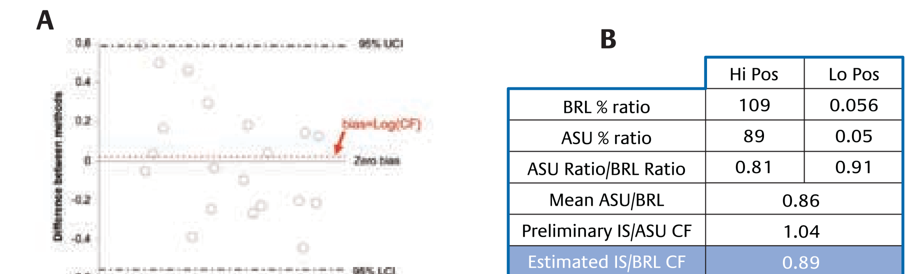


Figure 2. Data Processing and Reporting Workflow. (A) For each 96-well plate run, the ABI 7500 real-time instrument generates a data file containing batch run header information, sample ID, and the copy number of each target. **(B)** The BCR/ABL1 Quant XL analysis tool processes the raw data in Microsoft Excel and automatically generates 3 spreadsheets in a single workbook: processed standard curves (including graphic, R^2 and equation for each target), processed results (including sample ID, copy per RT for each target, ratio, % ratio and normalized BCR/ABL1 copy number) and source raw data for easy tracking and archiving (not shown). Each spreadsheet can be printed with automatic inclusion of the date and name of the raw data and processed files. **(C)** Example of result report generated at BRL. The report includes results for e1a2 fusion transcript, % ratio and normalized copy number of BCR/ABL1 per mL of blood. A description of the current estimated International Scale (IS) conversion factor and a definition of the Major Molecular Response (MMR) are also included. Historical data are reported in table and graphic formats.

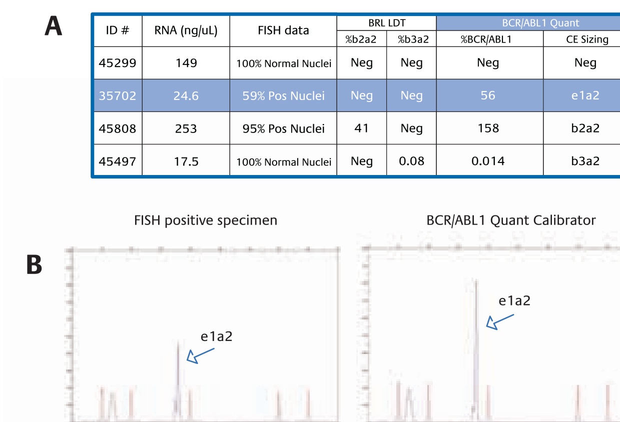
	b2a2	User 1 Day 1	User 1 Day 2	User 1 Day3	User 1 Average	User 1 %CV	User 2	User 3	Overall Average	Overall %CV
% Ratio Detected	1:100	5.67	7.23	5.68	6.19	14.52	4.20	4.77	5.51	20.85
% Ratio Detected	1:100,000	0.0061	0.0075	0.0093	0.0076	20.58	0.0029	0.0014	0.0054	59.26
Log (Δ Ratio)		2.97	2.99	2.79	2.91	3.75	3.16	3.53	3.08	9.06

Figure 3. Representative Inter-Assay Variability Results. A b2a2-positive cell line RNA was diluted into RNA from a translocation-negative cell line (HL-60) and tested in triplicate (1:100) or quadruplicate (1:100,000) by 3 different users on 5 different days. BCR/ABL1 to ABL1 % ratio were calculated by the XL analysis tool using independent standard curves generated in each of the 5 independent runs. Average and %CV for % Ratio and Log(Δ Ratio) are indicated for user 1 (3 runs) and overall (5 runs).



	Hi Pos	Lo Pos
BRL % ratio	109	0.056
ASU % ratio	89	0.05
ASU Ratio/BRL Ratio	0.81	0.91
Mean ASU/BRL	0.86	
Preliminary IS/ASU CF	1.04	
Estimated IS/BRL CF	0.89	

Figure 4. Establishing a Conversion Factor (CF) Relative to the International Scale (IS). The International Scale was established during the IRIS study (Hughes et al., N Engl J Med, 2003) and is anchored to a baseline BCR/ABL1 to control gene ratio (100% BCR-ABL[IS]) with a 3-log or more reduction indicative of Major Molecular Response (0.1% BCR-ABL[IS] or lower). A laboratory-specific Conversion Factor can be established by the sharing and testing of representative specimens between a given lab and an IS reference laboratory. (A) A preliminary Conversion Factor (CF) between BCR/ABL1 Quant and the IS was established by testing 20 RNA specimens at Asuragen and at the IMVS (Adelaide, Australia). The graph shows the Bland and Altman analysis used to determine the bias (0.017) between the 2 methods/labs. The corresponding calculated CF was 1.04, i.e. ASU ratio x 1.04 = IS ratio. (B) A CF of 0.89 for the BCR/ABL1 Quant assay validated at BRL was estimated by testing High and Low positive specimens in multiple runs at Asuragen and BRL. Establishment and validation of a laboratory-specific CF relative to the IS are further ongoing.



ID #	RNA (ng/uL)	FISH data	BRL LDT %b2a2	BRL LDT %b3a2	BCR/ABL1 Quant %BCR/ABL1	BCR/ABL1 Quant CE Sizing
45299	149	100% Normal Nuclei	Neg	Neg	Neg	Neg
35702	24.6	59% Pos Nuclei	Neg	Neg	56	e1a2
45808	253	95% Pos Nuclei	41	Neg	158	b2a2
45497	17.5	100% Normal Nuclei	Neg	0.08	0.014	b3a2

Figure 5. Resolution of FISH-Positive, LDT-Negative Results with BCR/ABL1 Quant. (A) Representative results for four remnant clinical specimens. The laboratory-developed tests (LDT) consist of simplex real-time quantitative RT-PCR assays for ABL1, b2a2 or b3a2 in a 96-well plate format (8 specimens maximum can be tested per plate). Sample #35702 was found positive by FISH but negative by LDT and would have required send out for analysis of e1a2 fusion transcripts. The sample was correctly identified as positive for e1a2 with the BCR/ABL1 Quant assay. **(B)** Representative examples of capillary electrophoresis traces. The qPCR reaction products can be directly analyzed by CE to identify e1a2-specific peaks at about 119 bp distinct from b2a2 (~90 bp) or b3a2 (~160 bp). Parallel analysis of the PCR products generated with the BCR/ABL1 Quant Calibrator Set serves as a positive e1a2 size control.

CONCLUSIONS

The BCR/ABL1 Quant assay (RUO)* has the advantage of both quantifying and distinguishing e1a2, b2a2, and b3a2 fusion transcripts in a single reaction. Streamlined reagent formulation, multiplex assay format, and inclusion of Armored RNA® external calibrators improve the workflow and increase the number of specimens that can be tested per run. Co-detection of ABL1 and inclusion of an exogenous ARQ process control enable reporting of BCR/ABL1 to ABL1 ratio for standardization to the International Scale as well as reporting of absolute BCR/ABL1 copy number. This format may contribute to enhanced patient care by preventing inaccurate results from potential inter-specimen or inter-run variability and by enabling inter-laboratory comparisons for different quantitative BCR/ABL1 testing and reporting methods.

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