Evaluation of Asuragen's qRT-PCR BCR/ABL Assay (RUO)* for the Quantitative Multiplex Detection of BCR/ABL1 (e1a2, b2a2, b3a2) and ABL1 Transcripts

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SUMMARY POINTS:

- Asuragen's BCR/ABL1 Quant Assay (RUO)* has a streamlined multiplex workflow allowing simultaneous detection and quantification of BCR/ABL1 (e1a2, b2a2, b3a2) and ABL1 transcripts by quantitative real-time RT-PCR technology.
- External and internal assay calibration is provided by Armored RNA® Quant™ technology and PCR products are compatible with downstream capillary electrophoresis analysis to determine fusion transcript identity.
- Analytical sensitivity reached at least 1:100,000 in this study by dilution of translocation-positive cell line RNA in HL-60 RNA or the equivalent of 10-50 copies of transcript.
- There was 100% agreement between the multiplex assay and singleplex laboratory-developed assays with a paired Pearson correlation of 0.976 for BCR/ABL1 to ABL1 copy number ratio in 38 residual clinical specimens covering a 5 Log dynamic range.

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 (Figure 1). The kit includes a calibrator set to generate three calibration curves and an exogenous spike in control to assess process efficiency, both built with Armored RNA® Quant™ (ARQ) technology (Figure 2). 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. The objective of this study was to evaluate the performance characteristics of the assay in a clinical laboratory setting and in comparison to an existing laboratory-developed test (LDT).

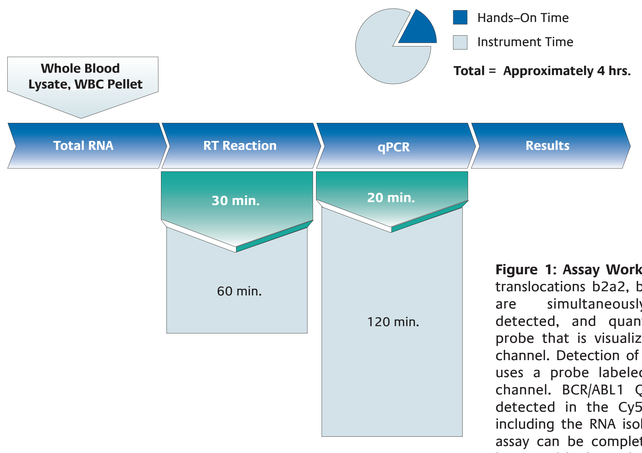


Figure 1: Assay Workflow. BCR/ABL1 translocations b2a2, b3a2, and e1a2 are simultaneously amplified, detected, and quantified using a probe that is visualized in the FAM channel. Detection of the ABL1 gene uses a probe labeled for the ROX channel. BCR/ABL1 Quant Norm is detected in the Cy5 channel. Not including the RNA isolation step, the assay can be completed in about 4 hours with less than 1 hour of hands-on time. Total RNA can be isolated with standard commercially available methods.

MATERIALS AND METHODS

Peripheral blood or bone marrow from leukemia patients were collected as part of standard clinical care and following a protocol approved by the Hospital of the University of Pennsylvania. Total RNA was isolated using the QIAamp RNA Blood Mini Kit (QIAGEN) according to the manufacturer's instructions. The laboratory-developed tests consisted of singleplex real-time quantitative RT-PCR assays for ABL1 or b2a2/b3a2 (both fusion transcripts are detected but not distinguished) and/or singleplex end point RT-PCR followed by gel analysis to identify e1a2, b2a2 or b3a2 fusion transcripts. Asuragen's BCR/ABL1 Quant (RUO) kit was evaluated using 51 archived total RNA samples following the Package Insert (500-2,000 ng RNA input recommended). Four (4) archived specimens were removed from the study for too low RNA concentration (<30ng/µL) and 2 for insufficient RNA volume. Briefly, 5 µL of RNA was reverse transcribed into cDNA and 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. For CE analysis, PCR products were diluted 1:100 and 1 uL was mixed with 1 uL of ROX ladder and 13 uL of HiDiTM 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 3130xl 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

Assay Calibration

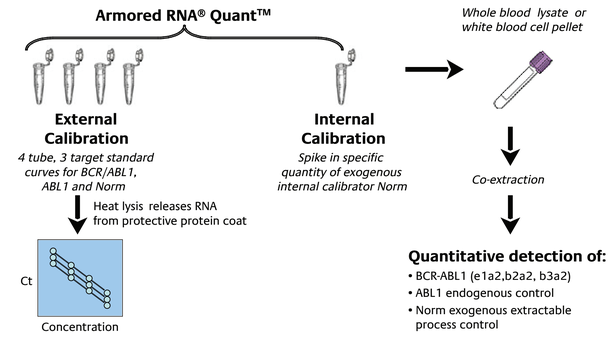


Figure 2: Assay calibration with Armored RNA Quant technology. The copy number of each target and the ratio of BCR/ABL1 to ABL1 copy number are calculated based on 3 calibration curves generated from only 4 multiplex qRT-PCR reactions. The 4 external calibrators consist of a blend of precisely quantified BCR/ABL1, ABL1 and Norm ARQs mixed at different concentrations (see Figure 3). In addition, a known fixed copy number of Norm ARQ can be spiked in the specimen lysate prior to RNA isolation (exogenous internal process control) for monitoring of process efficiency and absolute quantification of BCR/ABL1 (normalized BCR/ABL1 copies/mL of sample).

Analytical Performance

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

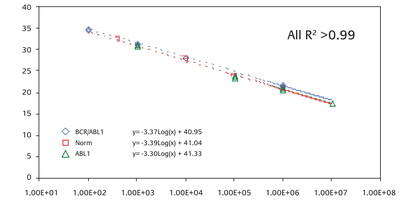


Figure 3: Representative examples of calibration curves. Following heat denaturation, the 4 ARQ calibrators were run in triplicate to evaluate repeatability. The resulting Ct (left) were automatically processed through the software to build 3 standard curves for BCR/ABL1, ABL1 and Norm (right) covering 5 Logs of copies per RT. R^2 were all >0.99.

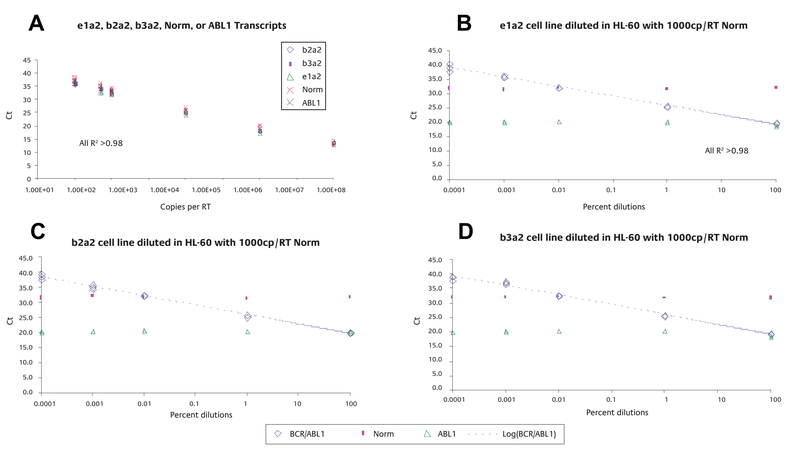


Figure 4: Analytical sensitivity. (A) Each of the 5 transcripts detected by the assay were prepared by in vitro transcription and individually tested in duplicate (1E+08, 1E+06, and 1E+04 copies), triplicate (100 and 50 copies) or quadruplicate (10 copies). In this study the dynamic range of the assay was ~7 Logs with a LOD of 10 copies and a LOQ of 50 copies. All transcripts were positively detected in their respective channels except 1 out of the 4 replicates for 10 copies of b3a2. R2 were all >0.98. **(B,C,D)** Samples were prepared by diluting total RNA purified from cell lines expressing e1a2, b2a2, or b3a2 fusion transcripts in a background of HL-60 RNA and keeping the total RNA input constant at 1,500 ng. About 1,000 copies of Norm transcripts were also spiked in each sample to mimic specimens purified in the presence of the Norm ARQ process control. Samples were tested in duplicate (100, 1 and 0.01%) or in triplicate (0.001 and 0.0001%). The linear dynamic range of the assay was ~6 Logs with 100% detection at 1:100,000 dilution (9/9). The same sensitivity was reached with 500-2,000 ng input of total RNA and all qPCR positive samples were also positive by CE analysis (data not shown).

Specimens

BCR/ABL1 Transcript	HUP LDT Data	Number of cases	Specimen Type	RNA (ng/µL)	Percent Agreement
Negative	Negative	3	2PB, 1BM	46-430	100%
Positive	Quantitative Only	4	3PB, 1BM	63-165	100%
	b2a2/b3a2 quantitative	38	36PB, 2BM	69-663	100%
Overall		45	41PB, 4BM	46-663	100%

Figure 5: Residual clinical specimens evaluated in this study. Archived total RNA from 45 patients was tested with the BCR/ABL1 Quant RUO assay. Three (3) specimens were negative with the laboratory-developed tests (HUP LDT), 4 were positive by end point PCR (qualitative data only) and 38 were positive by real-time qRT-PCR for b2a2 and/or b3a2 (the LDT can detect both fusion transcripts but does not distinguish between them). The negative and positive agreements between the LDT and the Asuragen assay in this study were 100%.
PB = Peripheral blood, BM = Bone marrow, CL = Cell Line.

Specimen ID	Specimen Type	RNA input (ng/RT)	Ct		Copies/RT		BCR/ABL1 to ABL1 Ratio	Log Ratio	Translocation by CE
			BCR/ABL1	ABL1	BCR/ABL1	ABL1			
421-6	PB	435	33.1	22.9	829	171,968	4.82E-03	-2.32	b3a2
819-1	PB	2,890	22.0	20.0	939,143	1,066,702	8.80E-01	-0.06	b2a2
697-4	BM	1,075	38.3	22.0	30	296,674	1.02E-04	-3.99	b3a2
678-3	PB	660	30.7	22.3	3,794	244,699	1.55E-02	-1.81	b2a2/b3a2
669-3	PB	1,645	37.1	21.6	65	391,833	1.67E-04	-3.78	b2a2
198-7	PB	1,190	ND	22.5	0	219,580	0	N/A	neg
b2 1:10	CL	1,000	23.9	20.2	283,461	955,182	2.97E-01	-0.53	b2a2
b2 1:100,000	CL	1,000	38.6	20.4	25	856,325	2.93E-05	-4.53	b2a2

Figure 6: Representative examples of BCR/ABL1 Quant RUO results. Total RNA specimens (5 µL) were reverse transcribed in 20 µL RT reactions and 5 µL was used per qPCR reaction. BCR/ABL1 and ABL1 copy numbers were calculated from 4 point multiplex ARQ standard curves run on the same plate. b2 1:10 and b2 1:100,000 are cell line (CL) dilution controls routinely used in the laboratory to assess run performance and confirm that a 4 Log decrease of BCR/ABL1/ABL1 is detected. PB=peripheral blood. BM=bone marrow.

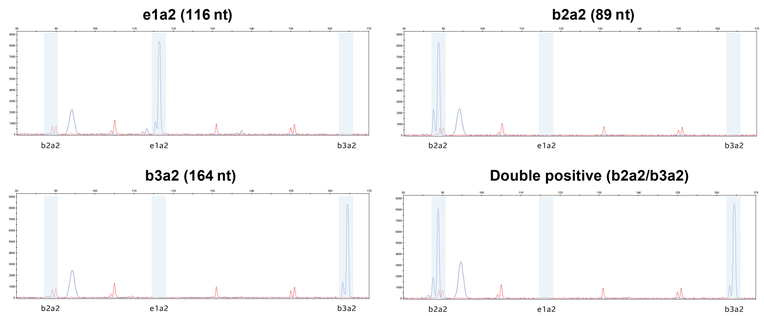
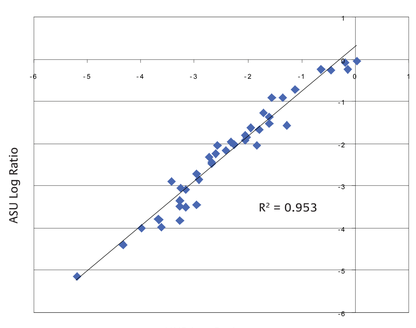


Figure 7: Representative examples of CE traces. Following real-time PCR, the amplification products were analyzed on an ABI 3130xl Genetic Analyzer using a 50 cm POP-7 capillary. Expected peak positions are highlighted.

Figure 8: Quantitative analysis. The paired Pearson correlation between the Log_{10} of BCR/ABL1 to ABL1 ratios obtained with Asuragen assay and the LDT was 0.976 using 38 specimens positive for b2a2 and/or b3a2 (5 specimens were also tested twice by different users and with different lots of Asuragen reagents for a total of 43 data points). For quantification, the Asuragen assay uses 4 point multiplex standard curves built with BCR/ABL1, ABL1 and Norm ARQ blends processed though the RT and qPCR steps while the HUP LDT uses a single RT with K562 total RNA followed by serial dilution of the cDNA into the qPCR reactions to build two independent 6 point standard curves for b3a2 and ABL1.



CONCLUSION

The BCR/ABL1 Quant RUO* assay has a streamlined workflow highly compatible with the molecular laboratory. Multiplex detection of all targets in a single well, preparation of single master mixes, minimal number of pipetting steps (10 including preparation of master mixes), and use of Armored RNA Quant technology to build 3 standard curves using only 4 reaction wells further reduce technologist time and increase the number of specimens tested per 96 well plate. In this study the assay has an analytical sensitivity of at least 1:100,000 with a linear dynamic range >6 Logs. Evaluation of 38 residual clinical specimens with b2a2/b3a2 quantitative data also showed an excellent correlation with existing laboratory-developed singleplex assays. Additional experiments are ongoing to evaluate the use of Norm ARQ as a process control for absolute quantification of BCR/ABL1 copy number and as a quality metric to track inter-specimen and inter-run performance.

** For Research Use Only. Not for Use in Diagnostic Procedures.*
The performance characteristics of this product have not been established.