Development of Armored RNA Quant® (RUO)* Reference Materials for the Standardization of Quantitative BCR/ABL1 Testing

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SUMMARY

- The use of Armored RNA Quant® (ARQ) technology to build stand-alone reference material sets for standardization of quantitative BCR/ABL1 testing was established.
- Robust manufacturing processes and stringent quality control specifications were developed for the production of BCR/ABL1 ARQ reference reagents.
- Further development and availability of Armored RNA Quant® (RUO)*secondary reference material sets will contribute to improved harmonization of BCR/ABL1 quantitative reporting.

INTRODUCTION

In chronic myeloid leukemia (CML) the molecular signature of BCR/ABL1 fusion transcripts provides a tool for monitoring of residual disease and potential recurrence during therapy. Currently, there are no certified BCR/ABL1 reference materials for monitoring of inter-run and inter-laboratory assay performance. ARQ technology is an *in vitro* RNA encapsidation system which produces homogeneous, nuclease-resistant, and analytically-quantified control reagents compatible with various RNA-based molecular assays. These characteristics present several advantages over the control materials currently used in residual disease monitoring assays, including in vitro transcribed RNA, cell line RNA or plasmid DNA, which all suffer from potential instability, lot-to-lot variability and/or an inability to monitor all of the assay steps. ARQ is already a well established technology in quantitative molecular infectious disease testing that could also fulfill the role of a stable, nuclease-resistant and consistently manufactured reference material in residual disease testing.

In 2007, a field study coordinated by the National Genetics Reference Laboratory (NGRL, Wessex, UK) was designed and conducted to evaluate the potential utility of ARQ technology as a reference material for standardization of BCR/ABL1 real-time quantitative RT-PCR testing methods. 29 different laboratories from over 11 different countries, using 14 different quantitative RT-PCR platforms, participated in the study. The study results provided preliminary support of ARQ reagents as a commutable secondary reference material. Here we report new advances towards the development of ARQ reference material sets for the standardization of quantitative BCR/ABL1 results reporting.

Sample	ABL1	BCR	GUS	b2a2 or b3a2	Ratio	% Ratio
Pos Level 1	30,000	30,000	30,000	30,000	1	100
Pos Level 2	30,000	30,000	30,000	3,000	0.1	10
Pos Level 3	30,000	30,000	30,000	300	0.01	1
Pos Level 4	30,000	30,000	30,000	30	0.001	0.1

Figure 1. ARQ blend formulations evaluated in the NGRL field trial study. The first prototype consisted of 4 levels of BCR/ABL1 to control gene ratios. The indicated copy numbers are per µL of formulated material.

MATERIALS & METHODS

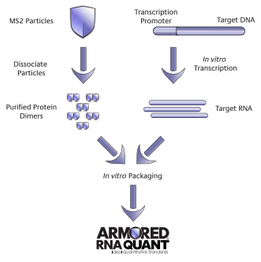
Synthetic RNA targets representing segments of the ABL1 and BCR control genes and BCR/ABL1 b2a2 and b3a2 fusion transcripts were prepared by in vitro transcription. Following packaging as ARQs, the copy number of each target was quantified using an NIST-traceable analytical phosphate assay. b2a2 or b3a2 ARQ blends covering 4 Logs were formulated in a constant background of ABL1 and/or BCR ARQs (Figure 1). Prototype sets were tested using quantitative RT-PCR assays previously calibrated against the International Scale (IS) by establishment of a Conversion Factor. Separately, a set of ARQ reagents were formulated and analyzed with Asuragen's BCR/ABL1 Quant RUO assay (Figure 3). Each sample was tested in 5 replicates on two independent runs.

RESULTS

ARQ Process Development

Figure 2. Armored RNA Quant Manufacturing and Quality Control. All steps of the manufacturing process (A) are performed in Asuragen's cGMP facility. Rigorous quality control methods and metrics **(B)** have been developed and validated to assess each key step in the manufacturing process.

A



B

Test	Method
Identity	Bi-directional sequencing of DNA template and gene-specific RT-PCR on IVT RNA intermediate and final ARQ-packaged RNA.
Integrity	Denaturing size analysis of IVT RNA intermediate and final ARQ-packaged RNA.
Purity	Protease on protein dimers. RNAse, DNase and residual DNA template contamination on IVT RNA intermediate. Protease, RNase, and DNase on formulation and storage buffer.
Quantity	Quantification of ARQ-packaged RNA copy number against an NIST-traceable standard. R^2 for standard curve >0.95 and %CV for replicate tests <20%.
Ratio	Functional testing of formulated ARQ blends using quantitative real-time PCR assays linked to the International Scale (e.g., Asuragen BCR/ABL1 Quant). R^2 for standard curve >0.98 and %CV for replicate tests <20-50% (depending on ratio).

A

Sample	ABL1	b2a2	b3a2	Ratio	% Ratio	
Pos Level 1	100,000	0	80,000	0.8	80	Log (Pos1/Pos2) = 3.2
Pos Level 2	100,000	50	0	0.0005	0.05	
Negative	100,000	0	0	0	0	

B

		BCR/ABL1		ABL1		Ratio			
		Ct	Cp/PCR	Ct	Cp/PCR	Replicate	AVG/Run	AVG/Level	
Pos Level 1 (b3a2)	Run 1	24.0	6.4E+04	21.9	7.7E+04	0.8225	0.9299	0.9272	
		23.7	7.9E+04	22.0	7.5E+04	1.0626			
		23.2	1.1E+05	21.4	1.1E+05	0.9938			
		23.2	1.1E+05	21.2	1.3E+05	0.8781			
		23.8	7.7E+04	21.8	8.6E+04	0.8923			
	Run 2	23.5	9.3E+04	21.6	1.0E+05	0.9295	0.9245		
		23.6	8.8E+04	21.7	8.9E+04	0.9838			
		23.2	1.1E+05	21.2	1.2E+05	0.8736			
		23.5	9.5E+04	21.6	1.0E+05	0.9446			
		23.7	8.2E+04	21.7	9.2E+04	0.8912			
Pos Level 2 (b2a2)	Run 1	33.6	94	21.6	9.8E+04	0.0010	0.0006	0.0005	Log(Pos1/Pos2) = 3.27
		34.7	44	21.5	1.1E+05	0.0004			
		34.3	56	21.2	1.2E+05	0.0004			
		33.9	73	21.1	1.3E+05	0.0005			
		34.1	66	21.5	1.1E+05	0.0006			
	Run 2	34.4	54	21.3	1.2E+05	0.0004	0.0004		
		34.8	40	21.3	1.2E+05	0.0003			
		34.6	45	21.3	1.2E+05	0.0004			
		34.7	41	21.3	1.2E+05	0.0003			
		34.7	42	21.6	9.5E+04	0.0004			
Negative	Run 1	ND	ND	21.3	1.2E+05				
		ND	ND	21.2	1.3E+05				
		ND	ND	21.3	1.2E+05				
		ND	ND	21.0	1.4E+05				
		ND	ND	21.3	1.2E+05				
	Run 2	ND	ND	21.2	1.3E+05				
		ND	ND	20.4	2.2E+05				
		ND	ND	21.1	1.4E+05				
		ND	ND	21.6	9.5E+04				
		ND	ND	21.3	1.2E+05				

C

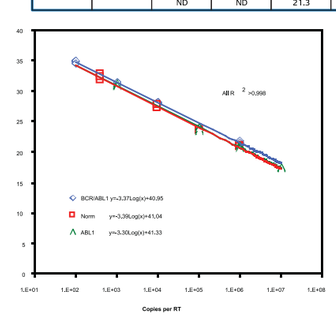


Figure 3: Evaluation of BCR/ABL1 to ABL1 ratio in formulated ARQ blends. Two Positive and one Negative ARQ blends **(A)** were formulated (copy number per µL) and tested with the BCR/ABL1 Quant RUO assay per the Package Insert. The assay is a multiplex real-time RT-PCR that can detect e1a2, b2a2, b3a2 and ABL1 with an analytical sensitivity and linear dynamic range appropriate to detect up to 5 Log variation in BCR/ABL1 to ABL1 ratio (for more information see Poster H21). Initial establishment of the assay Conversion Factor relative to the IS resulted in a CF of 1.04 (BCR/ABL1 Quant Ratio x 1.04 = IS ratio). Copy number per PCR **(B)** was determined relative to standard curves generated in triplicate with the BCR/ABL1 Calibrators provided with the kit **(C)**. As expected, %CV for the measured Pos Level 1 ratio (5 and 10%) were lower than for the Pos Level 2 ratio (13 and 37%).

Proposed Reference Material Design

Two stand-alone reference material sets have been proposed to the international BCR/ABL1 testing community, one for b2a2 and one for b3a2. Similar to the current international scale (IS) for quantitative measurement of BCR/ABL1, this quantitative reference material set would be anchored to a common baseline (100% BCR-ABL^{IS}) and major molecular response (0.1% BCR-ABL^{IS}). Each set would have the following features:

- 5 samples consisting of one BCR/ABL1 negative sample and 4 BCR/ABL1 positive samples.
- Each sample would contain 2 internal control targets (ABL1 and BCR) at a constant concentration level resulting in a control gene copy number per PCR reaction similar to representative clinical samples.
- The BCR/ABL1 positive samples would cover a range of BCR/ABL1 to control gene ratios (Reference Ratio or RR) that mimic the breadth of expression levels encountered in clinical specimens, including Complete Cytogenetic Remission (CCyR) and Major Molecular Response (MMR).
- Each sample would be ready-to-use for direct addition into the RT-PCR reaction after heat-lysis to release the target RNA from the protective protein coat. Alternatively, the samples could be used as external process controls by extracting the target RNA in parallel to clinical specimens through standard RNA isolation protocols.

				BCR/ABL1:ABL1		BCR/ABL1:BCR	
Sample	ABL1	BCR	b2a2 or b3a2	RR	% RR	RR	% RR
Pos Level 1	50,000	100,000	5,000	0.1	10	0.05	5
Pos Level 2	50,000	100,000	1,000	0.02	2	0.01	1
Pos Level 3	50,000	100,000	100	0.002	0.2	0.001	0.1
Pos Level 4	50,000	100,000	10	0.0002	0.02	0.0001	0.01
Negative	50,000	100,000	0	0	0	0	0

Figure 4: Reference Material Set Formulation Design. The indicated copy numbers are per µL of formulated, ready-to-use material. This formulation should be compatible with most RT and PCR protocols. For example, if 4 µL of reference material is used in a 20 µL RT reaction and 5 µL of the RT (cDNA) is transferred into the PCR (BCR/ABL1 Quant protocol), then 1 µL of the reference material ends up in the PCR and the number of copies in the table is equivalent to the number of copies present in the final PCR.

Figure 5. Reference Material Set Sequence Design. Schematic representation and exon numbers for the full b2a2 fusion transcript, the full BCR transcript (NM_004327.3), the full ABL1 transcript (NM_005157.3) and the b2a2, ABL1 and BCR ARQ-packaged RNA sequences. The ARQ b3a2 consists of exon 9-14 of BCR and exons 2-5 of ABL1.

International Survey Results

An online survey designed to gather feedback from the international BCR/ABL1 testing community was conducted by Asuragen in July-August 2009. The proposed design and a 2 part questionnaire were sent to about 150 labs worldwide. 64 labs participated and answered the single question in Part I. 31 labs further answered the questions provided in Part II of the survey. Answers to specific questions relevant to this poster regarding BCR/ABL1 assay designs currently implemented in those labs and feedback on the design/use of the proposed reference material sets are presented below.

Part I

Would the proposed designs for these reference material sets meet the needs of your labs?	
Yes	57 (89%)
No	7 (11%)

Part II

To be compatible with your assay's configuration, what endogenous internal control genes could be included in the reference set if different from those listed in Part 1?	
GUS	5
G6PDH	1
B2M	1

What material is currently routinely used in your lab as quality controls to monitor the acceptability of each run?	
Plasmid	9
Cell line RNA	22
IVT RNA	3
Other	6

What range of BCR-ABL1/control gene ratios is detected by your assay?	
3-4 logs	13 (42%)
>4Log	18 (58%)

If a quantitative reference material set anchored to the International Scale were available, would you continue to utilize the IS conversion factor established for your own assay?	
Yes	15 (48%)
No	16 (52%)

Which BCR/ABL1 fusion transcripts are routinely monitored in your lab?*	
e1a2	19 (61%)
b2a2	31 (100%)
b3a2	30 (97%)

*Five additional respondents indicated that they are considering adding e1a2 to their current testing menu.

What material is currently routinely used in your lab to build standard curves?	
Plasmid	25
Cell line RNA	6
IVT RNA	0
Other	1

What range of BCR-ABL1/control gene ratios should be represented by the reference material?	
3-4 Logs	22 (71%)
>4Log	9 (29%)

If a quantitative reference material set was available, how would you use it?	
Single test of each reference sample in each run	6 (19%)
Replicates of each reference sample in each run	16 (52%)
Only to monitor assay performance on a periodic basis	9 (29%)

CONCLUSIONS

The development of Armored RNA Quant® (RUO)* reference material panels will support the current international effort for standardization of quantitative BCR/ABL1 testing. The goal is to increase the confidence in BCR/ABL1 testing results, improve consistency in treatment decisions, and gather better data concerning the efficacy of approved or experimental treatments for leukemia patients. The commercial availability of these stable materials, manufactured under strict control and linked to an analytical, quantitative NIST standard and the International Scale, will ultimately benefit patients, physicians, diagnostic laboratories, and drug manufacturers by harmonizing the testing and reporting of BCR/ABL1 status.

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* Research Use Only. Not Intended for us in Diagnostic Procedures.