

SUMMARY POINTS

- A single-tube multiplex real-time PCR that simultaneously detects BCR/ABL, ABL and an exogenous internal calibrator (MRD Norm™) was developed.
- Five Armored RNA® Quant™ controls corresponding to 3 BCR/ABL targets, an endogenous internal target (ABL) and MRD Norm were developed.
- Demonstrated use of Armored Technology as an exogenous internal calibrator in a leukemia minimal residual disease assay.
- Established feasibility of using Armored Technology as a quantitative process control for calibration and standardization in molecular oncology testing.

INTRODUCTION

The introduction of Gleevec therapy for chronic myelogenous leukemia has resulted in increased interest in surveillance of disease-specific translocation transcripts. Such residual disease monitoring in oncology is often gauged by the quantitative measure of disease transcripts in patient samples. Different assay formats at different testing sites present challenges in comparisons of interlaboratory data. For example, nucleic acid testing is prone to sample loss and assay inhibition due to patient sample variability and suboptimal extraction yields. Positive controls often include either plasmid DNA, in vitro transcript RNA, or extracted cell line RNA. Unfortunately, these materials do not gauge the effects of patient sample processing leading to interlaboratory variation. The desire for an invariable endogenous normalizer has resulted in the widespread suggestion that such controls do not exist. Armored Technology is a method of creating homogeneous, consistent, nuclease-resistant control materials that can address these challenges. We describe the use of Armored Technology as quantitation standards and process controls for molecular oncology testing.

BACKGROUND

Armored RNA® is a proprietary technology invented and developed by Asuragen, Inc. and Cenetron Diagnostics (Austin, Texas) for protecting RNA from degradation by ribonucleases. One method for producing Armored RNA involves direct packaging of purified recombinant MS2 coat protein dimers with highly purified in vitro transcribed target RNA. This allows for the accurate quantitation of the packaged RNA using an established NIST (National Institute of Standards Technology) reference standard. The resulting purified RNA/protein complexes are protected from ribonuclease degradation and are stable in solution or human matrices at various temperatures and times. Furthermore, the RNA is easily released by heat, organic extraction or commercially available extraction kits commonly used in the isolation of nucleic acids from human specimens. As a result, Armored RNA can be stored for long periods of time without fear of degradation and can be directly used in molecular tests as both internal and positive controls, authentically mimicking patient specimens. Experiments showed no significant reduction in real time RT-PCR signal when Armored RNA Quant was incubated in human plasma at 4°C over 210 days or at 50°C for one hour (data not shown). Armored RNA Quant is therefore suitable as an accurate, reliable and durable quantitative standard in addition to being a qualitative assay control. Armored RNA technology has been integrated into several commercially available in vitro diagnostic kits. Established manufacturing processes following strict regulatory compliance have made Armored RNA a widely accepted positive control and standard for molecular diagnostic tests, new assay development and optimization, and laboratory training and qualification. Furthermore, spiking Armored RNA into clinical samples before RNA isolation enables monitoring of sample preparation efficiency. This provides a better control over the inherent variability between clinical labs, platforms and RNA isolation methods. The unique stability of Armored RNA after introduction into human matrices such as blood, plasma or bone marrow makes it an ideal extraction control allowing calibration of the entire process. Furthermore, individual Armored RNA preparations may be combined for use as a single-well control in multiplex assays. After formulating a mixture of Armored RNAs at levels appropriate for the downstream assay, the mixture can be extracted or heat lysed as described above.

MATERIALS AND METHODS

As a model for process controls and standards in molecular oncology monitoring, Armored RNA Quant controls were developed for BCR/ABL t(9;22) [b2a2, b3a2, e1a2], ABL (exon 10/11) and a b3a2-like exogenous internal calibrator sequence (MRD Norm). Target sequences were contained within a nuclease-resistant protein coat and quantified by a NIST-traceable phosphate assay. An extractable 3-target calibration set was used to generate 3 standard curves using only 3 wells: BCR/ABL, ABL and MRD Norm. The controls were extracted and detected using a multiplex qRT-PCR assay. Simultaneous detection of endogenous normalizer ABL and exogenous quantitation standard was achieved through the use of multiple dye-labeled probes.

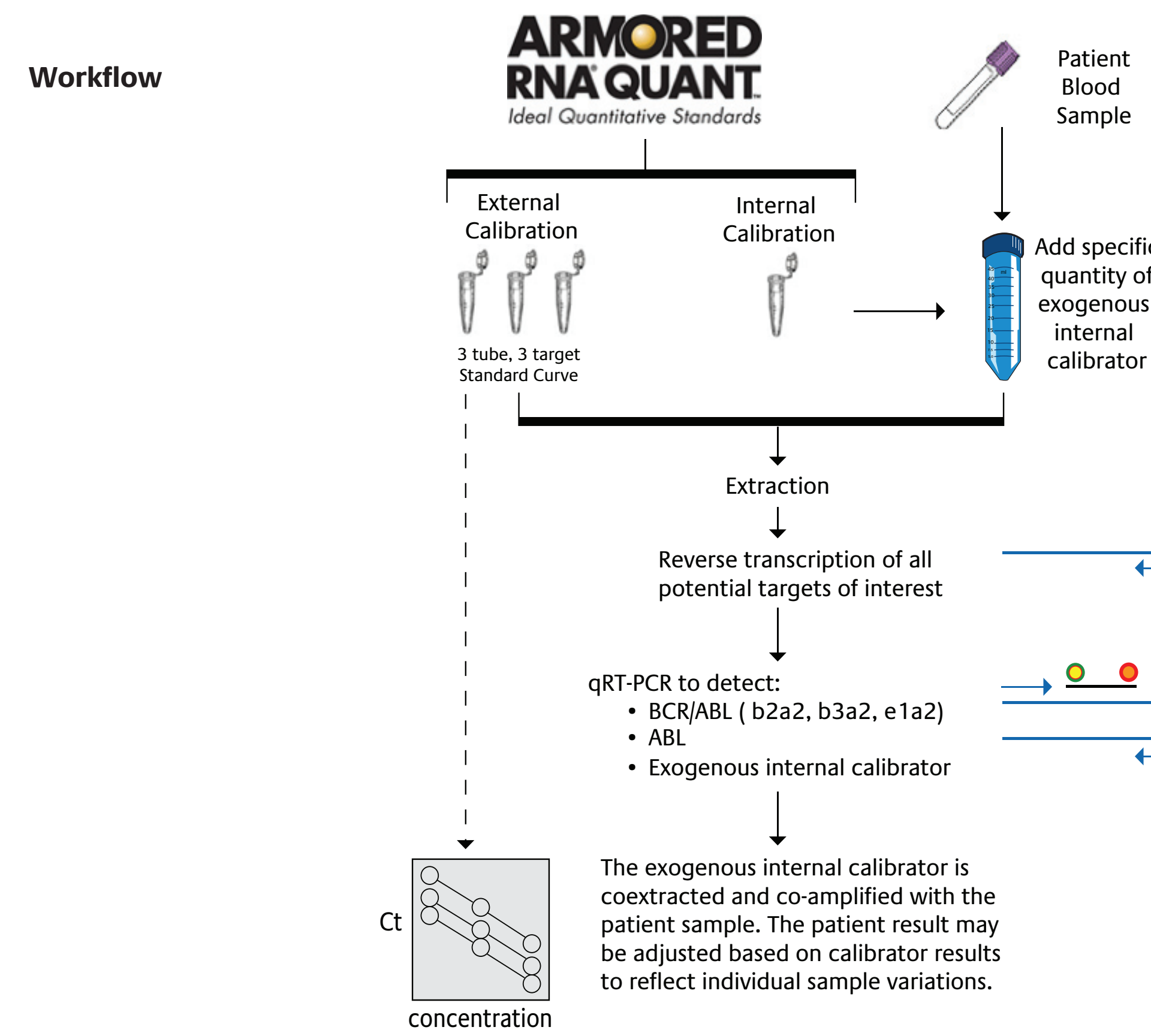


Figure 1. Proposed schematic of BCR/ABL detection workflow for single-tube multiplex qRT-PCR. Spiking Armored RNA into clinical samples before RNA isolation enables monitoring of sample preparation and amplification efficiencies. Individual Armored RNA preparations may be combined for use as a control in multiplex assays. After formulating a mixture of Armored RNAs at levels appropriate for the downstream assay, the mixture can be extracted. An example usage follows. After red blood cell lysis and white blood cell (WBC) enrichment by centrifugation, an Armored RNA Quant termed "MRD Norm" is added to the WBC pellet at a known amount, correlating to a particular copy number per ml of starting blood. Patient samples and a set of 3x3 Armored RNA Quant controls are then extracted using Trizol. Fluorescent dye was attached to the reverse primer of BCR/ABL target to allow optional post-PCR capillary fractionation to distinguish b2a2, b3a2 and e1a2 amplified products. Multiplex real-time PCR is completed in a single tube with the incorporation of dUTP to allow for amplicon carryover control with uracil-N-glycosylase (UNG). PCR products are amplified and detected on a Mx3005P real-time instrument (Stratagene). Three standard curves (BCR/ABL, ABL, and MRD Norm) are generated from the set of 3x3 Armored RNA controls. Data can be corrected for loss during extraction using MRD Norm.

Assay Design

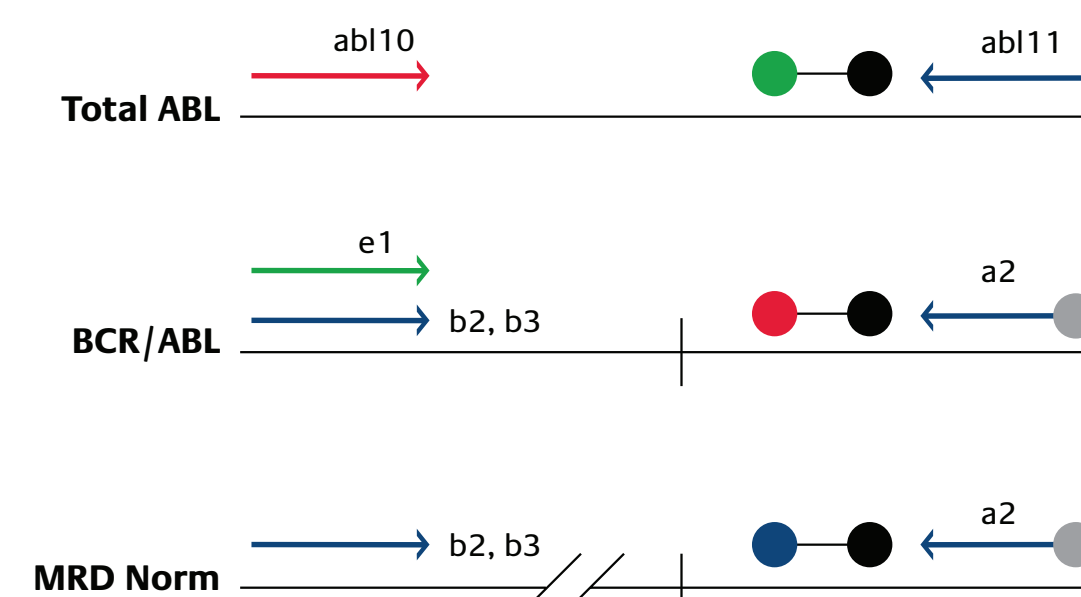


Figure 2. Primer and probe design. Primer and probe sequences targeting BCR/ABL and ABL were designed according to Cortes et al. (Ref 1). MRD Norm was designed by subtracting several nucleotides from the b3a2 target sequence and randomizing the probe region. As in Cortes et al., a fluorescent dye was attached at the 5' end of the a2 reverse primer to give an optional size differentiation.

RESULTS

Verification of Assay Design

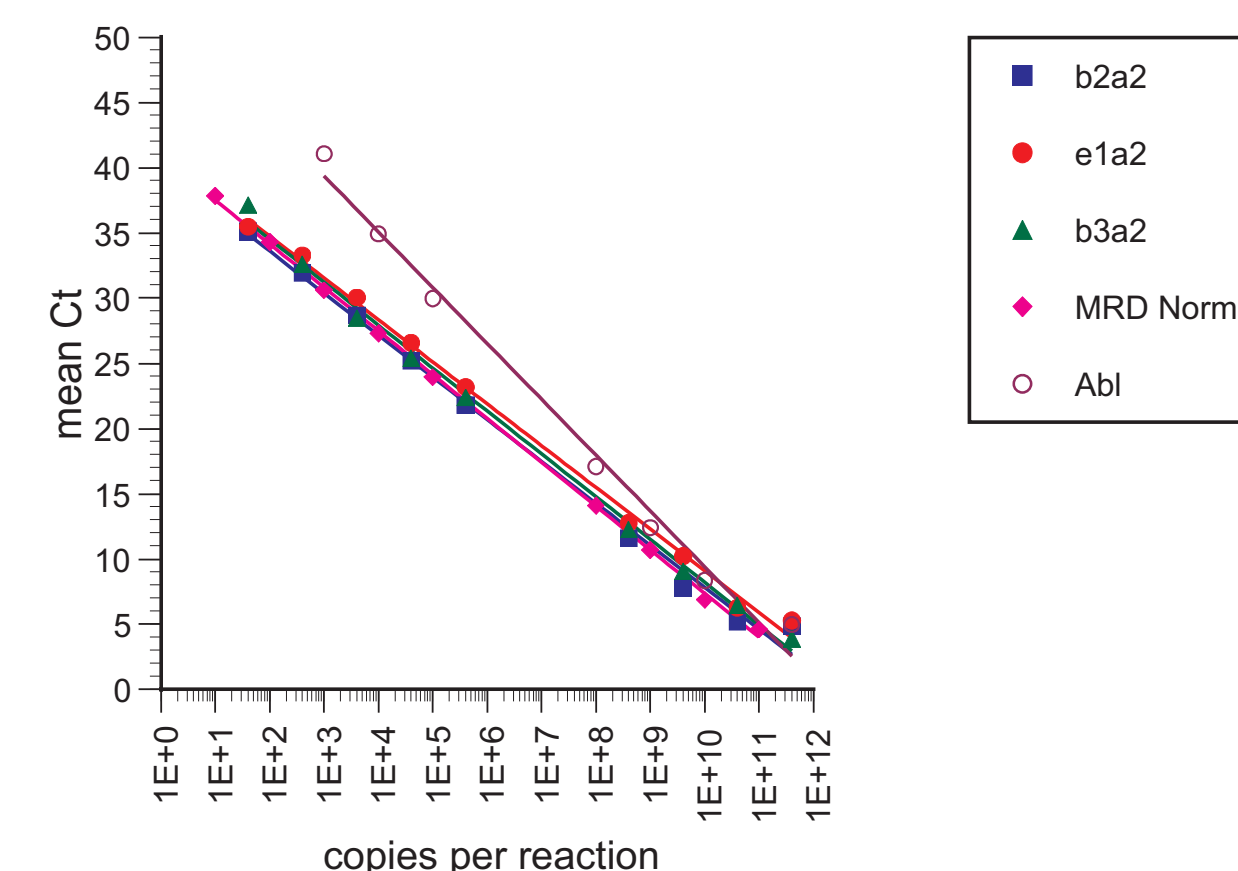


Figure 3. Assay linearity and analytical sensitivity using IVTs (in vitro transcripts). After assay optimization, serial dilutions of BCR/ABL b2a2, b3a2, e1a2, ABL and MRD Norm IVTs were individually tested for linear range and analytical sensitivity of each target in the assay.

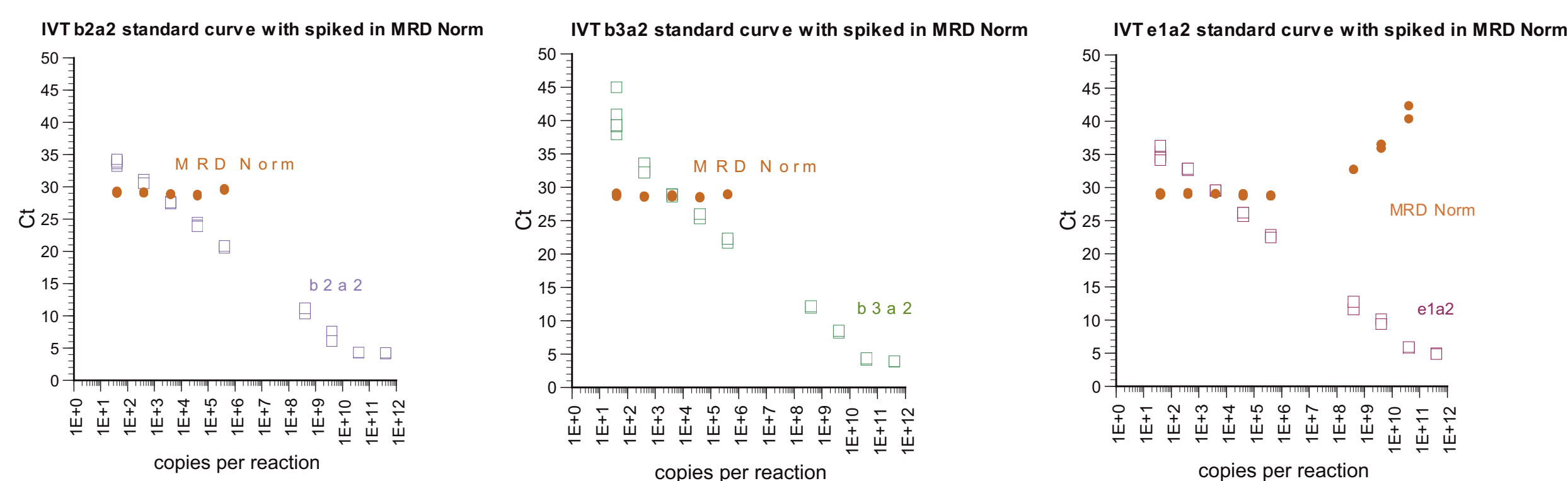


Figure 4. Linear curves of BCR/ABL with constant levels of MRD Norm IVTs (in vitro transcripts). To introduce MRD Norm as an external control, 1000 copies of MRD Norm IVT were spiked into each IVT dilution. The spiked MRD Norm did not interfere with target detection. If the input RNA of BCR/ABL was higher than 1.0E+06, MRD Norm detection was not observed (b2a2 and b3a2) or was less efficient (e1a2), presumably because high target levels outcompeted the MRD Norm internal control.

Development of Quantitative Multiplexed Control Sets

	1	2	3
Armored e1a2	High (5.0E+10 copies/ml)	Medium (5.0E+07 copies/ml)	Low (1.0E+06 copies/ml)
Armored MRD Norm	Medium (5.0E+06 copies/ml)	Low (5.0E+05 copies/ml)	High (5.0E+07 copies/ml)
Armored ABL	Low (1.0E+07 copies/ml)	High (5.0E+10 copies/ml)	Medium (5.0E+07 copies/ml)

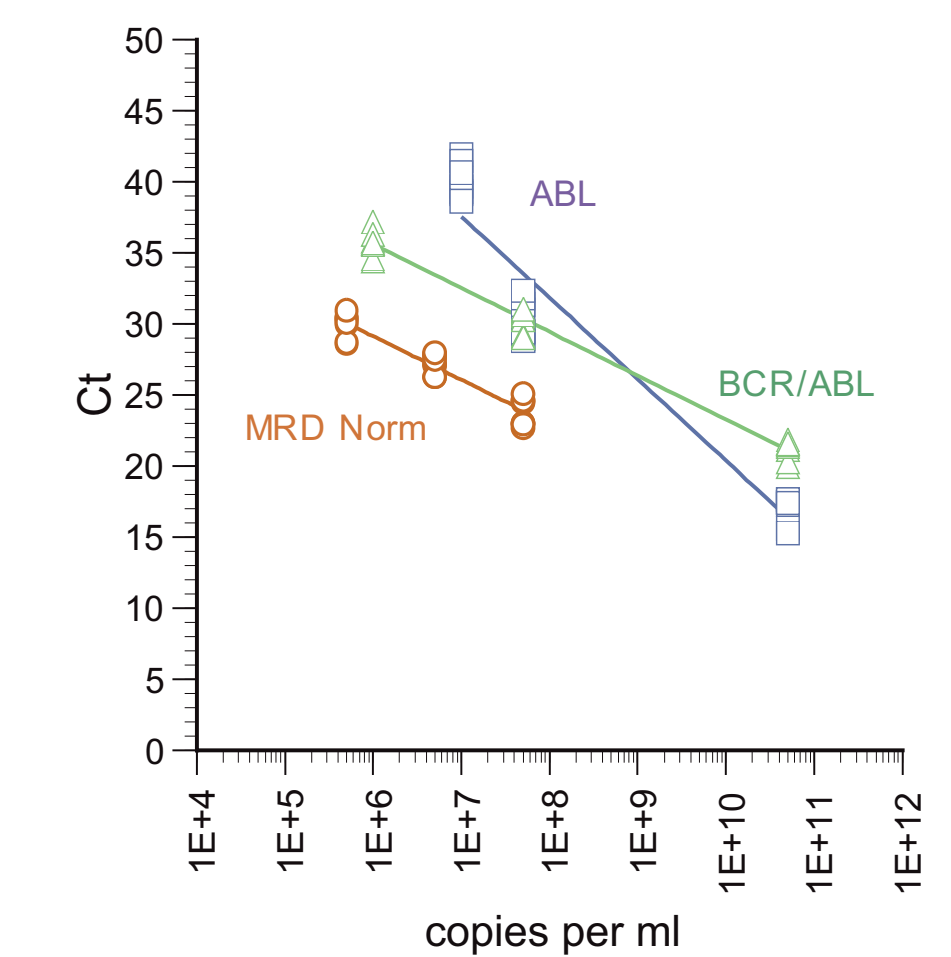


Figure 5. Standard curves generated from 3x3 Armored controls. A three-target calibration set was used to generate 3 standard curves using only 3 wells: BCR/ABL, ABL and MRD Norm. These preliminary results demonstrated the ability to use Armored RNA technology as extractable, quantitative controls.

BCR/ABL copy number				
Samples	Input	Average output	Stdev	%Recovery
Standard 1	1.0E+09	9.8E+05	5.3E+05	0.1
Standard 2	1.0E+06	1.6E+03	1.0E+03	0.2
Standard 3	2.0E+04	3.7E+01	1.4E+01	0.2

ABL copy number				
Samples	Input	Average output	Stdev	%Recovery
Standard 1	2.0E+05	3.8E+02	2.0E+02	0.2
Standard 2	1.0E+09	8.6E+07	5.2E+07	8.6
Standard 3	1.0E+06	7.4E+04	4.7E+04	7.4

MRD Norm copy number				
Samples	Input	Average output	Stdev	%Recovery
Standard 1	1.0E+05	9.0E+03	4.3E+03	8.6
Standard 2	1.0E+04	1.6E+03	9.9E+02	15.7
Standard 3	1.0E+06	9.5E+04	5.4E+04	9.5

Table 1—Percent recovery after Trizol extraction. Armored RNA Quant e1a2, ABL, and MRD Norm were formulated in a background of TSM III buffer with 10 ng/μl of polyA carrier RNA. Input quantities were based on an established NIST (National Institute of Standards Technology) reference standard and phosphate assay. Output quantities were based on the qRT-PCR assay described above in this work.

Use of an Armored RNA as a Quantitative Process Control

Samples	MRD Norm copy number		
	input	output	% recovery
donor1	4.0E+04	8.0E+03	19.9
donor2	4.0E+04	7.0E+03	17.5
donor3	4.0E+04	9.1E+03	22.7
donor4	4.0E+04	7.4E+03	18.4
donor5	4.0E+04	8.4E+03	20.9
donor6	4.0E+04	9.7E+03	24.2
donor7	4.0E+04	7.7E+03	19.3
donor8	4.0E+04	1.1E+04	27.3
donor9	4.0E+04	9.1E+03	22.7

Table 2—Percent recovery after Trizol extraction. Total RNA was extracted from 5 ml of blood from nine healthy donors using Trizol. Armored RNA Quant MRD Norm was spiked into the white blood cells pellets at the same amount in all donors. Four different amounts of b2a2 Armored Quant were spiked into the white blood cell pellets of donors 1-4.

Samples	Spiked-in BCR/ABL	Copy per reaction			%Ratio	Copy per ml blood	
		BCR/ABL	ABL	%Ratio		BCR/ABL	Normalized BCR/ABL
donor1	4.8E+04	1.2E+03	2.5E+05	0.5	2.4E+03	1.2E+04	
donor2	4.8E+05	1.2E+04	2.5E+05	4.9	2.4E+04	1.4E+05	
donor3	4.8E+06	9.6E+04	1.0E+05	95.1	1.9E+05	8.4E+05	
donor4	4.8E+07	1.1E+06	4.3E+04	2443.1	2.1E+06	1.2E+07	
donor5*	N/A	6.2E+00	2.9E+05	0.0	1.2E+01	5.9E+01	
donor6*	N/A	4.3E+01	2.5E+05	0.0	8.6E+01	3.6E+02	
donor7*	N/A	5.9E+01	3.5E+05	0.0	1.2E+02	6.1E+02	
donor8*	N/A	3.6E+01	2.5E+05	0.0	7.2E+01	2.6E+02	
donor9*	N/A	8.9E+00	3.0E+05	0.0	1.8E+01	7.8E+01	

Table 3. Percent of BCR/ABL Ratio and copy per ml blood of BCR/ABL obtained from IVT standard curves. A widely used method for reporting of BCR/ABL levels is as percent ratio to ABL levels; however, it cannot correct for differences between laboratories. An alternative method of reporting is copy per ml blood. ARQ MRD Norm can be added to each patient sample prior to extraction allowing for more consistent interlaboratory resulting. The normalized copy per ml of BCR/ABL was proportional to the spiked BCR/ABL. Without normalization, copy per ml of BCR/ABL did not account for the loss during extraction. The relative amount of BCR/ABL in the WBC of healthy individuals ranges from 5-20 copies per 5.0E+07 to 1E+08 WBC. The frequency of healthy individuals having the BCR/ABL in their blood is 30% (22/73) and is age dependent (Bieraux et al). The range of BCR/ABL of donors 5-9 obtained from our assay ranges from 59 to 610 copies per 4 to 11E+06 WBC.

CONCLUSION

Armored RNA Quant controls were generated for three BCR/ABL targets, an endogenous internal target and an exogenous internal quantitation standard. Armored Technology allowed the development of a single tube BCR/ABL assay that simultaneously used both internal and external calibration in a manner that mimics the effects of patient sample processing. In addition, non-target Armored nucleic acids could act as sample-to-sample process controls when added to each patient sample prior to extraction, allowing for more consistent interlaboratory resulting.

Acknowledgments

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