Development and Validation of Highly Sensitive MRDx BCR-ABL Test for Monitoring Deep Molecular Response in Patients with Chronic Myeloid Leukemia

Abstract #: 2617

Julie A. Toplin, Kristy A. Drafahl, Jonathan M. Eibl, Clark C. Fjeld, Thomas D. Yager, Staci A. McAdams, Kevin D. Hawkins, and Chad D. Galderisi, MolecularMD Corp., Portland, OR

Introduction

Treatment of chronic myeloid leukemia (CML) patients with tyrosine kinase inhibitors (TKI) has led to progressively lower levels of disease burden and higher rates of complete molecular responses. Very few assays with an analytical sensitivity of 4.5-logs (0.00316% BCR-ABL/ABL) have been validated, and assay standardization at these levels has been problematic (EUTOS, 2007). In order to have an International Scale (IS) standardized assay that can precisely and accurately detect 4.5 log reductions in BCR-ABL/ABL ratios, the test system design must be tightly controlled to ensure this critical performance metric can be reproducibly met. The MolecularMD MRDx BCR-ABL Test was developed and validated to be an accurate, reproducible, and highly sensitive IS-standardized solution to measure minimal residual disease (MRD) in CML patients using either PAXgene Blood RNA or EDTA blood collection tubes.

Test Overview

The MolecularMD MRDx BCR-ABL Test is a quantitative real-time polymerase chain reaction (RT-qPCR) test that provides for accurate and sensitive quantitation of BCR-ABL transcripts (e13a2/b2a2 or e14a2/b3a2) and ABL transcripts in RNA extracted from peripheral blood samples that have been collected from CML patient blood specimens (Figure 1). Total RNA is extracted from peripheral blood and serves as a template for RT-qPCR. The test is performed using a one-step RT-qPCR protocol wherein the reverse transcription and quantitative real-time PCR reactions are performed in the same well. BCR-ABL and ABL amplicons are generated and detected in real-time using TaqMan MGB probes. Quantitation is achieved using RNA calibration standards and linear regression analysis. BCR-ABL transcript levels are measured in relation to the ABL transcript as an endogenous reference. The BCR-ABL/ABL ratio is calculated and converted to the International Scale. Calibrators are assayed for both BCR-ABL and ABL in every run alongside patient samples. RNA controls provided at 3-log major molecular response (MMR) and 4.5-log molecular response (MR4.5) allow for on-plate verification of test accuracy and reproducibility over this critical transcript range. The integrated conversion factor provides test results on the International Scale.

Figure 1: MRDx BCR-ABL Test Sample Processing

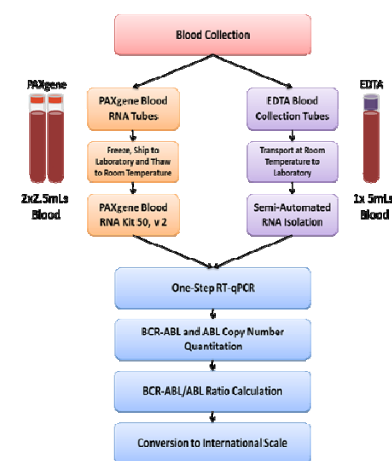


Table 1: Lot to Lot Testing of Enzyme Systems

Sample Tested	Enzyme Lot Testing-Candidate Enzyme (Standard Deviation)	Enzyme Lot Testing-Final Enzyme (Standard Deviation)
IVT Calibrator 3e5 copies	0.565	0.144
IVT Calibrator 3e2 copies	0.580	0.129
IVT Calibrator 3e1 copies	0.636	0.222
IVT Calibrator 3e0 copies	1.052	0.571

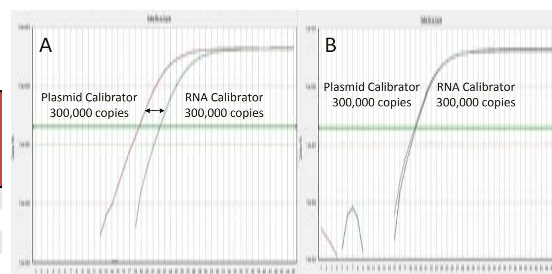
Test Development

To achieve a sensitivity of 4.5 logs, several one-step enzyme systems were evaluated. The enzyme systems were tested on plasmid and RNA calibrators with the same copy numbers to evaluate the efficiency of the reverse transcription (C(t)) for plasmid and RNA calibrators (Figure 2, brown and green curves respectively). An enzyme system with low RT efficiency has delayed C(t) values for plasmid calibrators compared to RNA calibrators (Figure 2A, highlighted by the black arrow). Only enzyme systems with overlapping plasmid and RNA calibrators (Figure 2B) were chosen to be evaluated for sensitivity and precision.

The final enzyme system chosen for the MRDx BCR-ABL Test was the system with the highest reverse transcription efficiency and the best sensitivity and precision as determined by testing three manufactured lots (Table 1).

In addition, in order to better control for the variation of the reverse transcription, *in vitro* transcribed RNA calibrators were developed to create a standard curve for copy number determination rather than the more commonly used plasmid calibrators that may not accurately reflect the same processing as the patient sample RNA.

Figure 2: Example Enzyme Systems-Performance on Plasmid and RNA Calibrators



For Further Information please contact info@molecularmd.com or visit www.molecularmd.com.

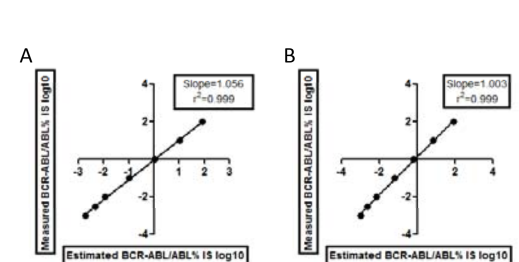
Analytical Validation—Linearity

A series of samples was created by diluting total RNA from CML patients into RNA extracted from normal healthy donor blood samples (Table 2). The samples with the following dilutions were analyzed from both PAXgene RNA blood collection tubes and EDTA blood collection tubes: 10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, $10^{-4.5}$, and 10^{-5}. The mean BCR-ABL/ABL % IS ratios and $\log_{10}$ BCR-ABL/ABL % IS ratios are shown below for each dilution series. The results of the linear regression analysis done by comparing the approximate $\log_{10}$ BCR-ABL/ABL ratio to the $\log_{10}$ mean BCR-ABL/ABL % IS are shown in Figure 3. The assay has been shown to be linear over at least 4.5 logs (r^2>0.98) for both RNA extracted from blood collected in PAXgene RNA blood collection tubes (4.642 logs) and EDTA blood collection tubes (4.955 logs) (Table 2). The range of the assay expressed in the BCR-ABL/ABL ratio % IS has been shown to be 82.4% to 0.00184% for the PAXgene RNA dilution series and 84.8% to 0.000955% for the EDTA RNA dilution series.

Table 2: Assay Linearity in PAXgene and EDTA Blood tubes

Dilution	Approximate BCR-ABL/ABL %	Sample Type	Mean BCR-ABL/ABL % IS	Mean ($\log_{10}$) BCR-ABL/ABL % IS
10^{0}	100	PAXgene RNA	82.4	1.92
10^{-1}	10	PAXgene RNA	10.2	1.01
10^{-2}	1	PAXgene RNA	1.04	0.0153
10^{-3}	0.1	PAXgene RNA	0.105	-0.980
10^{-4}	0.01	PAXgene RNA	0.0113	-1.95
$10^{-4.5}$	0.0032	PAXgene RNA	0.00459	-2.34
10^{-5}	0.001	PAXgene RNA	0.00184	-2.73
10^{0}	100	EDTA RNA	84.8	1.93
10^{-1}	10	EDTA RNA	6.80	0.833
10^{-2}	1	EDTA RNA	0.656	-0.183
10^{-3}	0.1	EDTA RNA	0.0648	-1.19
10^{-4}	0.01	EDTA RNA	0.00685	-2.16
$10^{-4.5}$	0.0032	EDTA RNA	0.00224	-2.65
10^{-5}	0.001	EDTA RNA	0.000955	-3.02

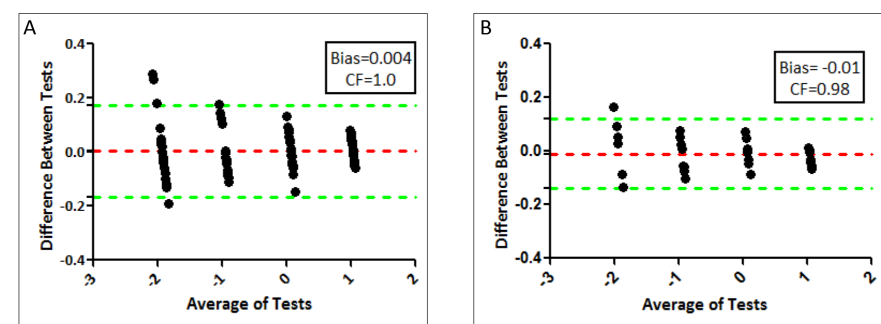
Figure 3: Linear Regression Analysis for PAXgene Blood RNA Samples (A) and EDTA Blood Samples (B)



Analytical Validation—Alignment to the International Scale

The MRDx BCR-ABL Test reports on the international scale with a conversion factor of 1.0 by use of the WHO International Standard 1st WHO International Genetic Reference Panel for quantitation of BCR-ABL translocation by RQ-PCR; secondary standards were created for bi-annual monitoring of the assay conversion factor to ensure accurate reporting of patient results on the IS. Per the NIBSC suggested protocol for running the 1st WHO International, on each of 5 different days, a different WHO primary panel of 4 freeze-dried materials were reconstituted and extracted. The rehydrated/extracted WHO Primary samples and the BCR-ABL (IS) Secondary Standards were tested in duplicate using the MRDx BCR-ABL Test over ten days using one lot of reagents. The study was repeated and the data combined (Figure 4A). Confirmation of the conversion factor on a small subset of samples was done by an independent laboratory using different real-time PCR instruments and multiple technicians (Figure 4B).

Figure 4: Bland-Altman Bias Plots for IS Conversion Factor – MolecularMD Data Set (A) and Independent Laboratory Confirmation(B)



Analytical Validation—Precision and Limit of Detection

The limit of detection (LOD) for blood drawn into PAXgene Blood RNA tubes was validated by testing a dilution series created from a baseline CML patient blood sample diluted into non-diseased subject blood. Creating this type of dilution series allows for the determination of the LOD in the actual clinical sample matrix as opposed to using cell line dilution series that have much higher BCR-ABL and ABL copy numbers than routine patient specimens. Based on the analysis of each level of sample tested over a multi-day, multi-operator, and multi-instrument study (Table 3), the LOD of the MRDx BCR-ABL Test was determined to be MR4.7 (Mean=MR4.9, 95% CI=MR5.2 to MR4.7) using the more conservative upper bound of the 95% confidence interval. The precision was evaluated based on the standard deviation of the $\log_{10}$ BCR-ABL/ABL ratio and was found to be ≤0.20 SD at MR5.0 and above. The LOD for blood drawn into EDTA blood collection tubes was validated by creation of a dilution series from a baseline CML patient RNA sample diluted into RNA isolated from blood of non-diseased subjects (Table 4). Based on the number of samples with detectable BCR-ABL in at least 95% of replicates over a multi-day, multi-operator, and multi-instrument study, the LOD of the MRDx BCR-ABL Test was determined to be MR4.9 (Mean=MR5.0, 95% CI=MR5.1 to MR4.9) using the more conservative upper bound of the 95% confidence interval with precision being ≤0.22 SD at MR5.0 and above.

Table 3: Limit of Detection and Precision for PAXgene Blood RNA Tubes

Log Sensitivity	Mean BCR-ABL/ABL Ratio (log)	Standard Deviation	Mean BCR-ABL/ ABL Ratio (%)	Standard Deviation	Sample Size	% Detected
-1.4	0.623	0.0144	4.20	0.140	5	100
-2.5	-0.537	0.0399	0.291	0.0262	10	100
-3.6	-1.59	0.0399	0.0259	0.00474	20	100
-4.1	-2.08	0.112	0.00851	0.00219	20	100
-4.6	-2.65	0.193	0.00246	0.000950	20	100
-5.0	-3.01	0.205	0.00104	0.000523	20	90

Table 4: Limit of Detection and Precision for PAXgene Blood RNA Tubes

Log Sensitivity	Mean BCR-ABL/ABL Ratio (log)	Standard Deviation	Mean BCR-ABL/ ABL Ratio (%)	Standard Deviation	Sample Size	% Detected
-0.1	1.96	0.0453	84.8	8.58	27	100
-1.2	0.862	0.0398	6.80	0.623	27	100
-2.2	-0.153	0.0418	0.656	0.0646	27	100
-3.2	-1.16	0.0616	0.0648	0.00927	27	100
-4.2	-2.14	0.107	0.00685	0.001560	27	100
-4.6	-2.65	0.160	0.00224	0.000835	27	100
-5.0	-3.04	0.216	0.000955	0.000484	27	96

Analytical Validation—Accuracy

In addition to being calibrated to the international scale, the accuracy of the BCR-ABL copy numbers, ABL copy numbers, and BCR-ABL/ABL ratio using the MRDx BCR-ABL Test was were compared to droplet digital PCR (ddPCR). A one-step BCR-ABL ddPCR assay was developed with different primers and probes than the MRDx BCR-ABL Test, and the two assays were used to analyze ten patient samples and a series of RNA controls from MR1 to MR5. For the BCR-ABL copy numbers, ABL copy numbers, and the BCR-ABL/ABL ratio, the bias of the MRDx Test relative to ddPCR by Bland-Altman method analysis was 0.045, 0.0329 and 0.046 respectively. The BCR-ABL and ABL copy numbers in addition to the resulting BCR-ABL/ABL ratio (%) and fold difference between the two methods are shown in Table 5.

Table 5: Accuracy by Comparison to droplet digital PCR

Sample ID	BCR-ABL Copies/µg ddPCR	BCR-ABL Copies/µg MRDx	ABL Copies/µg ddPCR	ABL Copies/µg MRDx	BCR-ABL/ABL Ratio (%) ddPCR	BCR-ABL/ABL Ratio (%) MRDx	Fold Difference
F186	86	63	3.20E+05	3.57E+05	0.0269	0.0177	1.5
F187	656	681	3.64E+05	3.86E+05	0.180	0.176	1.0
F188	2160	1700	2.74E+05	2.38E+05	0.788	0.715	1.1
F190	96	97	3.66E+05	3.79E+05	0.0263	0.0257	1.0
F192	109	132	5.40E+05	4.21E+05	0.0201	0.0313	1.6
F193	23	25	8.88E+05	7.22E+05	0.0026	0.0034	1.3
F195	66	63	4.78E+05	3.97E+05	0.0139	0.0158	1.1
F196	36	24	4.84E+05	4.03E+05	0.0075	0.0061	1.2
F199	26	14	2.52E+05	2.68E+05	0.0103	0.0052	2.0
F201	616	659	3.60E+05	3.60E+05	0.1711	0.1830	1.1
MR1 RNA	20112	20553	1.70E+05	1.77E+05	11.8456	11.5901	1.0
MR2 RNA	1979	1828	1.66E+05	1.74E+05	1.1901	1.0527	1.1
MR3 RNA	217	182	1.58E+05	1.65E+05	0.1369	0.1100	1.2
MR4 RNA	20	15	1.69E+05	1.76E+05	0.0119	0.0086	1.4
MR4.5 RNA	5	4	1.64E+05	1.71E+05	0.0033	0.0023	1.5

Conclusions

Based on the validation data, the MRDx BCR-ABL Test is an accurate, reproducible, and highly sensitive IS-standardized solution to the growing need for a reliable and robust quantitative BCR-ABL assay that can be used for the monitoring of minimal residual disease in CML patients.