Development of a synthetic secondary standard for the quantification of p210 *BCR-ABL1* standardized to the International Scale (IS)

Ariella Danziger, Emma Farrell, Joan Gordon, Matthew Held, Molly Kephart, Loren Krott, Daniel Magoon, Tania Spenlinhauer

Maine Molecular Quality Controls, Inc., Saco, Maine, USA

Introduction

Philadelphia (Ph) chromosomal rearrangements consisting of *BCR-ABL1* t(9;22) (q34;q11) are the hallmark of Chronic Myeloid Leukemia (CML) and Acute Lymphoblastic Leukemia (ALL). Ph translocations are present in >95% of CML, 5% of pediatric ALL, and 10-25% of adult ALL. Current recommendations for monitoring CML patients call for measuring the levels of *BCR-ABL1* standardized to the International Scale (IS) to ensure harmonized reporting across laboratories.

A WHO traceable reference standard was developed and validated to monitor the quantitative detection of *BCR-ABL1* to *ABL1* using numerous quantitative RT-qPCR assays to create assay specific correction factors that enable standardized reporting on the %IS to provide confident disease monitoring.

Methods

The Birlinn™ BCR-ABL1 p210 IS Panel C230 consists of a range of *BCR-ABL1* RNA transcript concentrations mixed with a fixed concentration of *ABL1* RNA transcript, to produce five p210 levels; 0.0032%, 0.01%, 0.1%, 1%, and 10%. Three lots of the Birlinn BCR-ABL1 p210 IS Panel were tested alongside 5 panels of WHO Standards using the REALQUALITY RQ-BCR-ABL p210 One-Step assay (AB ANALITICA s.r.l). Linear Regression and lot-specific Correction Factors (CF) were calculated and a WHO-traceable %IS value was assigned to each level, according to NIBSC Instructions for Use[1].

The Birlinn BCR-ABL1 p210 IS Panel was tested across multiple reagent lots and at 3 sites to confirm accuracy in reporting. Additional testing was also performed using Qiagen's *ipsogen*® BCR-ABL1 Mbcr IS-MMR DX kit and Bioclarma SensiQuant p210 Master Mix.

Intra-Lot Precision

Testing 5 levels of 1 lot of Birlinn BCR-ABL1 p210 IS Panel 6 times on the same REALQUALITY RQ-BCR-ABL p210 One-Step assay reagent lot, all reported % ratios were within the expected ranges on (**Table 1**) and %CV for Cp values of each target (*BCR-ABL1* and *ABL1*) are ≤10% (**Table 2a and 2b**).

Table 1. Average % Ratio for each level of one lot of Birlinn BCR-ABL1 p210 IS Panel One lot of 0.0032%, 0.01%, 0.1%, 1%, and 10% levels were tested six times on a single lot of REALQUALITY RQ-p210 BCR-ABL1 One-Step.

Level	Lot #	% reported ratio
REF 5	F29SEP23A	0.0032
REF 4	G29SEP23A	0.010
REF 3	H29SEP23A	0.104
REF 2	J29SEP23A	1.24
REF 1	K29SEP23A	9.6

Table 2. Mean, standard deviation and %CV for report *BCR-ABL1* (2a) and *ABL1* (2b) for each level of one lot of Birlinn BCR-ABL1 p210 IS Panel One lot of 0.0032%, 0.01%, 0.1%, 1%, and 10% levels were tested in six times on a single lot of REALQUALITY RQ-p210 BCR-ABL1 One-Step.

2a

Level	Lot #	Mean (Ct)	STDV	%CV
REF 5	F29SEP23A	21.3	0.38	1.78
REF 4	G29SEP23A	21.1	0.19	0.90
REF 3	H29SEP23A	21.4	0.12	0.56
REF 2	J29SEP23A	21.6	0.11	0.49
REF 1	K29SEP23A	21.3	0.20	0.95

2b

Level	Lot #	Mean (Ct)	STDV	%CV
REF 5	F29SEP23A	35.7	0.54	1.52
REF 4	G29SEP23A	33.9	0.52	1.54
REF 3	H29SEP23A	30.6	0.23	0.74
REF 2	J29SEP23A	27.1	0.34	1.26
REF 1	K29SEP23A	23.8	0.19	0.78

Inter-lot Precision

Three lots of Birlinn BCR-ABL1 p210 IS Panel were tested across 18 days, incorporating three reagent lots and multiple operators. A total of 270 samples were tested in duplicate across 21 plates (*20 samples from each level for lot #1, and 17 samples from each level for lots #2 and 3*) **(Table 3a and 3b)**.

Table 3a. Mean, STDV and %CV for Cp values of *BCR-ABL1* and *ABL1* and % ratios for three lots tested across 3 reagent lots. Lot #1 n=20, Lot #2 n=17 and Lot #3 n=17 per level (Total of 270 samples).

	BCRABL1 Cp values					ABL1 Cp values				
	REF 5	REF 4	REF 3	REF 2	REF 1	REF 5	REF 4	REF 3	REF 2	REF 1
Mean	36.0	34.4	31.0	27.6	24.3	21.9	21.9	21.9	21.8	21.7
STDV	0.7	0.5	0.5	0.4	0.5	0.5	0.5	0.5	0.5	0.5
%CV	2.0	1.5	1.5	1.6	2.0	2.2	2.3	2.2	2.1	2.4

Table 3b. Mean % ratios for each lot tested across 3 reagent lots. Lot #1 n=20, Lot #2 n=17 and Lot #3 n=17 per level (total of 270 samples).

	REF 5	REF 4	REF 3	REF 2	REF 1
% Ratio Mean	0.0042	0.012	0.123	1.20	10.5

To confirm equivalency of batch-to-batch, data from testing 3 lots of Birlinn BCR-ABL1 p210 IS Panel on the same day, same plate using one reagent lot, and by same operator was analyzed. The %CV for Cp values of each target (*BCR-ABL1* and *ABL1*) (**Table 4**) were less than the %CV for the average Cp values reported when tested across multiple reagent lots, plates and days (**Table 3a**).

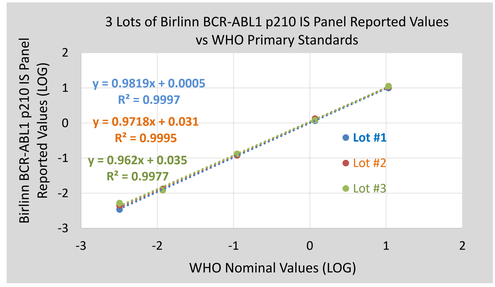
Table 4. Mean, STDV and %CV for Cp values of *BCR-ABL1* and *ABL1* and % ratios for three lots tested across 1 reagent lots.

	BCRABL1 Cp values					ABL1 Cp values				
	REF 5	REF 4	REF 3	REF 2	REF 1	REF 5	REF 4	REF 3	REF 2	REF 1
Mean	35.6	33.9	30.6	27.2	23.8	21.3	21.4	21.3	21.4	21.0
STDV	0.3	0.1	0.2	0.2	0.1	0.2	0.1	0.1	0.2	0.5
%CV	0.8	0.2	0.6	0.7	0.5	1.0	0.3	0.6	1.1	2.2

Linearity and WHO-Traceability

Linear regression was applied to each of the three lots of Birlinn BCR-ABL1 p210 IS Panel to confirm a parallel relationship to the WHO Primary Standard Nominal values (**Figure 1**).

Figure 1. Linear regression of the Log Reported values vs the WHO Primary Standard Nominal Values.



A linear relationship between observed and expected values over the range of the secondary standards enables a lot specific correction factor to be applied to the mean reported values for each of the lots of secondary standards to derive values on the IS (**Table 5**).

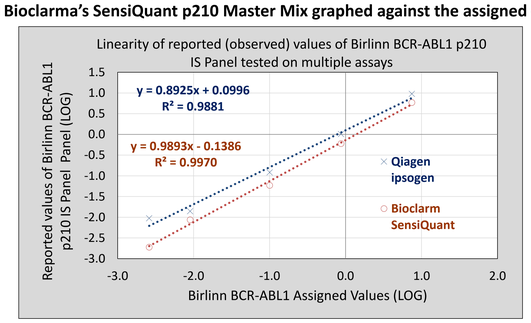
Table 5. Assigned values calculated by applying lot-specific CF to the mean reported values of each of the three lots of secondary standards. Lot-Specific CF values were calculated using the reciprocal of the anti-log 10 of the intercept of the linear regression for the observed % ratios (reported) for each of the WHO samples graphed against the WHO Nominal (expected) values. Uncertainties were calculated as the SQRT[(WHO Primary STDV)^2 + (Secondary Standard STDV)^2].

WHO Nominal Values	Lot #1 Assigned (+/- uncertainties)	Lot #2 Assigned (+/- uncertainties)	Lot #3 Assigned (+/- uncertainties)
10.7469	7.46 (+/- 1.71)	8.36 (+/- 1.94)	9.36 (+/- 2.91)
1.1672	0.87 (+/- 0.18)	1.04 (+/- 0.34)	0.99 (+/- 0.34)
0.1112	0.10 (+/- 0.04)	0.09 (+/- 0.03)	0.11 (+/- 0.04)
0.0118	0.009 (+/- 0.006)	0.010 (+/- 0.004)	0.010 (+/- 0.004)
0.0032	0.0026 (+/- 0.0012)	0.0034 (+/- 0.0014)	0.0043 (+/- 0.0016)

Testing across Multiple RT-qPCR Assays

The Birlinn BCR-ABL1 p210 IS Panel was tested using *ipsogen*® BCR-ABL1 Mbcr IS-MMR DX kit (QIAGEN) and SensiQuant p210 Master Mix (Bioclarma) demonstrating linearity with R^2 values greater than 0.98 (**Figure 2**) and reported %IS values within expected ranges based on the assigned value uncertainties (**Table 6**).

Figure 2. Linear regression of the reported values when tested with Qiagen's *ipsogen*® BCR-ABL1 Mbcr IS-MMR DX kit and Bioclarma's SensiQuant p210 Master Mix graphed against the assigned values.



A linear relationship between the observed values and assigned values over the range of the secondary standards enables the calculation of a %IS CF. A CF is calculated as the reciprocal of the anti-log10 of the intercept of the linear regression and the reported values were converted to %IS.

Table 6. Reported %IS values of Birlinn BCR-ABL1 p210 IS Panel on two RT-qPCR assays. %IS values are based on generating assay specific CF using the reported (observed) values against the assigned (expected values).

Assigned Value	ipsogen	SensiQuant
0.0026 (+/- 0.0012)	0.0076	0.0026
0.009 (+/- 0.006)	0.011	0.012
0.10 (+/- 0.04)	0.10	0.08
0.87 (+/- 0.18)	0.82	0.83
7.46 (+/- 1.71)	7.65	8.15

Clinical Site Testing

Linearity and accurate reporting were observed with data from three external clinical sites using the REALQUALITY RQ-p210 BCR-ABL1 One-Step assay as the testing method (**Figure 3 and Table 7**).

Figure 3. Linearity of one lot of Birlinn BCR-ABL1 p210 IS Panel tested at multiple clinical site locations. Each site tested all levels in duplicate across 4 plates (n=8/level).

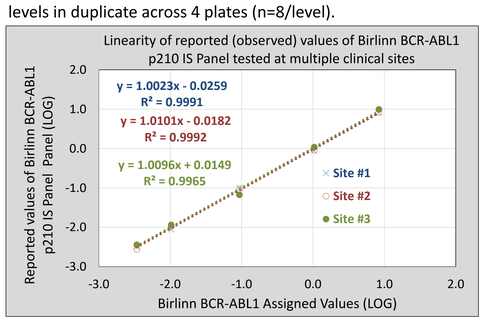


Table 7. Reported values of Birlinn BCR-ABL1 p210 IS Panel when tested at multiple clinical sites.

Assigned Value	Site #1	Site #2	Site #3
0.0034 (+/- 0.0014)	0.0032	0.0028	0.0036
0.010 (+/- 0.004)	0.009	0.010	0.012
0.09 (+/- 0.03)	0.10	0.09	0.07
1.04 (+/- 0.34)	0.88	0.91	1.09
8.36 (+/- 1.90)	8.14	8.36	9.91

Conclusions

- A linear relationship was found between reported and assigned values for all levels of the reference standards when tested across three different BCR-ABL RT-qPCR assays, enabling the calculation of an assay specific correction factor to allow harmonized reporting on the International Scale (%IS).
- The BCR-ABL p210 Panel was validated for accuracy, precision, robustness and traceability and can be used as a WHO traceable reference standard to create assay specific correction factors to enable standardized reporting on the International Scale (%IS).

References:
[1]1st WHO International Genetic Reference Panel for Quantitation of BCR-ABL Translocation, NIBSC code: 09/138 Instructions for use (Version 4.0, Dated 13/1).
[2]ISO17511 Second Edition, 2020-4