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Title

Standardization of molecular monitoring for chronic myeloid leukemia in Latin America using locally produced secondary cellular calibrators

Running title

Latin America RT-qPCR standardization platform

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Residual disease in chronic myeloid leukemia (CML) patients undergoing therapy with tyrosine kinase inhibitors (TKIs) is measured by assessing the quantity of transcripts of the BCR-ABL1 fusion gene in peripheral white blood cells[1]. This analysis is based on reverse-transcription quantitative PCR (RT-qPCR) technology; however, the wide array of methods used worldwide has led to large variation in quantitative BCR-ABL1 measurements, which hamper inter-laboratory comparative studies[2,3]. It is now recognized that monitoring BCR-ABL1/control gene ratios on the International Scale (IS) is vital for the management of patients with CML[4]. Efforts to harmonize procedures to measure BCR-ABL1 fusion transcripts have included important investments in sample exchange programs to derive laboratory-specific conversion factors (CF); these efforts showed improvements in inter-laboratory concordance rates, but the process is laborious and limited due to the lack of a common set of reference samples that can be shared on a global scale. This requirement was recently addressed in part by the formulation and validation of the first World Health Organization (WHO) International Genetic Reference Panel for quantitation of BCR-ABL1 by RT-qPCR[5]. The WHO primary standards consist of a 4-level panel of e14a2-positive lyophilized cell line dilutions. Each level has an assigned IS-value, which was obtained by repeated testing of each sample level in expert IS-standardized laboratories. Unfortunately, the stock of WHO primary standards is limited, and their accessibility has been restricted to manufacturers of testing kits or secondary reference standards. In this study, we aimed to develop and validate secondary reference materials calibrated to the IS through the WHO primary standards in order to facilitate standardization of molecular monitoring in Latin America.

The study design comprised five principal steps as illustrated in Supplementary Figure 1. The study was conducted by a single reference laboratory (rLAB, Buenos Aires, Argentina), which initially obtained a CF to the IS by sample exchange with the reference laboratory in Adelaide in 2010. To ensure consistent performance of rLAB analytical system (MolecularMD, OR, USA), we included 2 quality control (QC) RNA-samples with a high and low BCR-ABL1 level in each run

(Supplementary Table 1 and Supplementary Material and Method, Section 1). To further validate our method prior to calibration of secondary reference materials (see below), we derived a CF by using WHO primary standards (NIBSC code 09/138) from the United Kingdom National Institute for Biological Standards and Control (Potters Bar, UK). The antilog of the estimated mean bias (-0.152) was designated as the conversion factor (CF=0.7) for the rLAB method (Supplementary Table 2), very close (i.e. well within 2 fold) to the value of 0.45 obtained by sample exchange 3 years previously.

Previous local exploratory investigation indicated us that RT-qPCR methodologies harmonization was necessary since the tests were inadequately comparable, considering that the three acceptance criteria proposed by Muller *et al.*[6] were not satisfied (Supplementary Tables 3 and 4). To this aim we established and validated five batches of cellular calibrators produced by serial dilution of the Ph-positive cell line K562 in the Ph-negative cell line HL-60. Formulations were planned to target IS% ratios close to each of the established TKI clinical response criteria[7,8,9], that is, between 10%, 1%, 0.1% ($MR^{3.0}$), 0.01% ($MR^{4.0}$). An additional dilution (0.001%, $MR^{5.0}$) was included in order to assess the limit of detection of the methods and was not considered for the estimation of the CF (Figure 1). Cell mixes were stabilized by lyophilisation (Supplementary Figure 2A); assignment of IS%-values to each batch and level of these secondary standards was achieved by repeated testing of randomly picked ampoules on 4 non-consecutive days (Figure 1 and Supplementary Material and Method, Section 2). Stability studies of the freeze-dried cells, showed no significant changes in the BCR-ABL1/ABL1 ratio over time at different temperatures up to six months (Supplementary Figure 2B). The calibrated secondary reference standards were distributed to 18 testing laboratories from 7 countries in Latin America. Each laboratory followed the calibrators' instructions for use, which recommended 4 independent runs on different days for the two panels (Supplementary Materials and Methods, Section 3). A total of 1,312 RT-qPCR positive results were generated; mean raw percentage ratios generated in all laboratories were consistent with a 10-fold serial dilution and were linear for the first 4 levels (Supplementary Figure 3); prior to CF calculation, in order to assess if bias was uniform across the BCR-ABL1 expression range, Bland-Altman analysis[10] was performed for each assay (Supplementary Material and Method, Section 4). All the assays showed a uniform bias, indicating that a valid CF could be calculated for these methods (Supplementary Table 5). All raw %ratios measured within the linear range of each local RT-qPCR method were compared against a single set of reference values, the nominal IS%-ratios. Laboratory-specific CFs were calculated by Bland–Altman method[10] (Supplementary Table 5). The relative mean bias ranged from -0.45-fold to +0.25-fold in distinct laboratories (Figure 2A); after correction of the individual raw percent ratios with the laboratory specific CF, the residual mean bias was null (Figure 2B). In addition, raw BCR-ABL1/ABL1 %ratios generated in each laboratory were used to assess the corresponding level-specific coefficient of variation (CV): between 8% and 58% (12 out of 18 laboratories with an average CV less than 30%). As expected, BCR-ABL1 was inconsistently detected below the linear quantitative detection range of the RT-qPCR method in the lowest positive samples (fifth calibrator); for the 10 laboratories that tested the fifth calibrator, the overall BCR-ABL1 detection rate ranged from 12.5% to 100%. In total, eight out of ten laboratories could reproducibly detect BCR-ABL1 in level-5 samples (Supplementary Table 5). Interestingly, 5 laboratories had previous standardization, thus we could compare the new CF to the laboratory's current CF; when no methodology modifications were introduced since the time of the last calibration we obtained highly concordant CFs (Supplementary Table 6).

For appropriate clinical decisions, we need to judge agreement between the methods after IS conversion; to this aim, we interchanged 41 whole blood samples (divided into two parts) from CML patients with Lab#09. The concordance in MR^{IS} between the reference method and the external laboratory was 88% after conversion (36 out of 41 samples were in the same MR category) (Figure 2C). This result underlines the importance of conversion to the IS, given that, after harmonization we were able to halve the number of discordant data (from 9 to 5 cases).

In the present study, we show that secondary reference biological calibrators anchored to the WHO primary standards can decrease inter-laboratory variability. Our results, together with those recently reported by Cross *et al.*[11], substantiate the objective initially set during the establishment of the WHO primary standards, that is, to facilitate worldwide diffusion of the IS. For the first time in Latin America, this study provides a platform on which to assess the performance of distinct clinical BCR-ABL1 tests and confirm the utility of secondary reference materials to further improve IS accuracy and inter-laboratory precision. This effort will continue in the future by providing secondary reference material to the centres involved in this project and potential new participants; moreover, due to its higher precision and absolute quantification capability, we are evaluating to include digital PCR as the calibration method for the future.

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Supplementary Information is available at Leukemia's website

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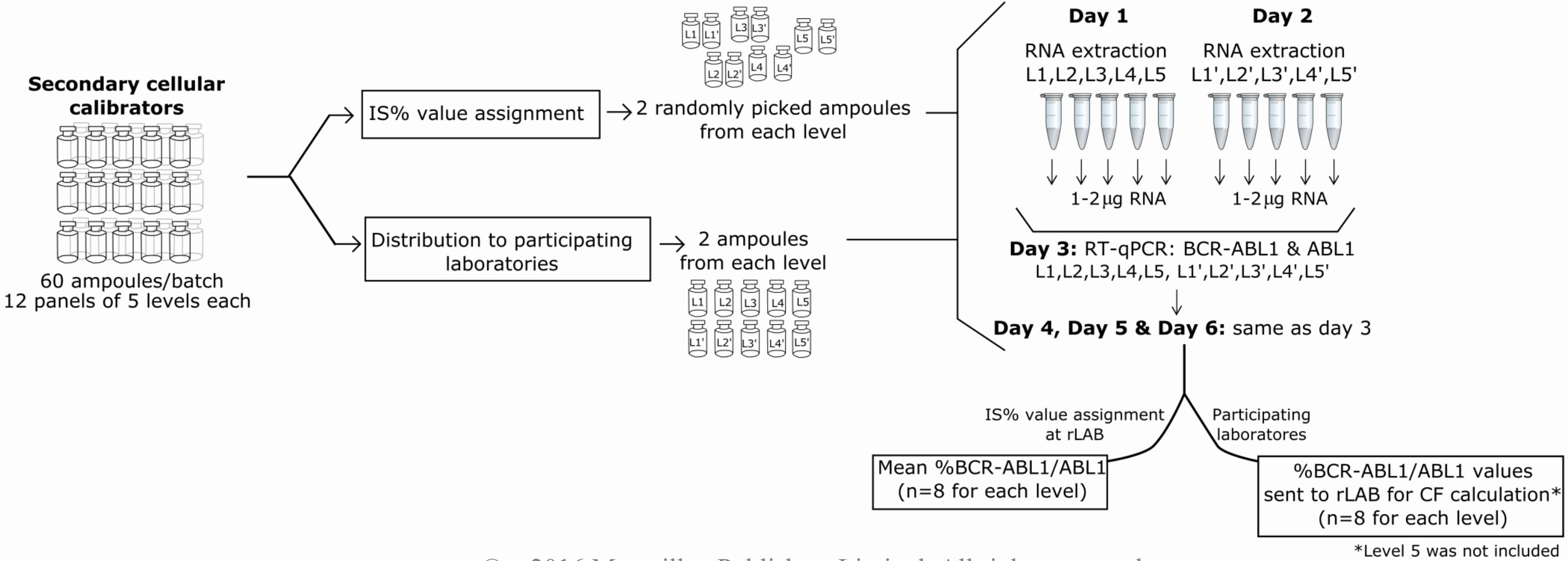
Figure legends

Figure 1: Study design for the nominal IS% value assignment and BCR-ABL1 testing by participating laboratories.

Figure 2: (A) Relative difference between nominal and measured IS% ratios for all calibrator levels combined (black circle). (B) Residual difference after CF-conversion. Error bars show 95% LOA interval. (C) Correlation graph. Comparison of IS%-ratios from 41 whole blood samples, interchanged between the rLAB and local Lab#09. Grey-coloured squares represent the concordance area for the assignment of the molecular response. Black points; concordant results. Red points; discordant results. Black line; equality line.



Secondary cellular calibrators
60 ampoules/batch
12 panels of 5 levels each
IS% value assignment
2 randomly picked ampoules from each level
L1 L1' L3 L3' L5 L5' L2 L2' L4 L4'
Distribution to participating laboratories
2 ampoules from each level
L1 L2 L3 L4 L5
L1' L2' L3' L4' L5'
Day 1
RNA extraction
L1,L2,L3,L4,L5
Day 2
RNA extraction
L1',L2',L3',L4',L5'
1-2µg RNA
1-2µg RNA
Day 3: RT-qPCR: BCR-ABL1 & ABL1
L1,L2,L3,L4,L5, L1',L2',L3',L4',L5'
Day 4, Day 5 & Day 6: same as day 3
IS% value assignment at rLAB
Participating laboratores
Mean %BCR-ABL1/ABL1
(n=8 for each level)
%BCR-ABL1/ABL1 values sent to rLAB for CF calculation*
(n=8 for each level)
*Level 5 was not included

Figure 1

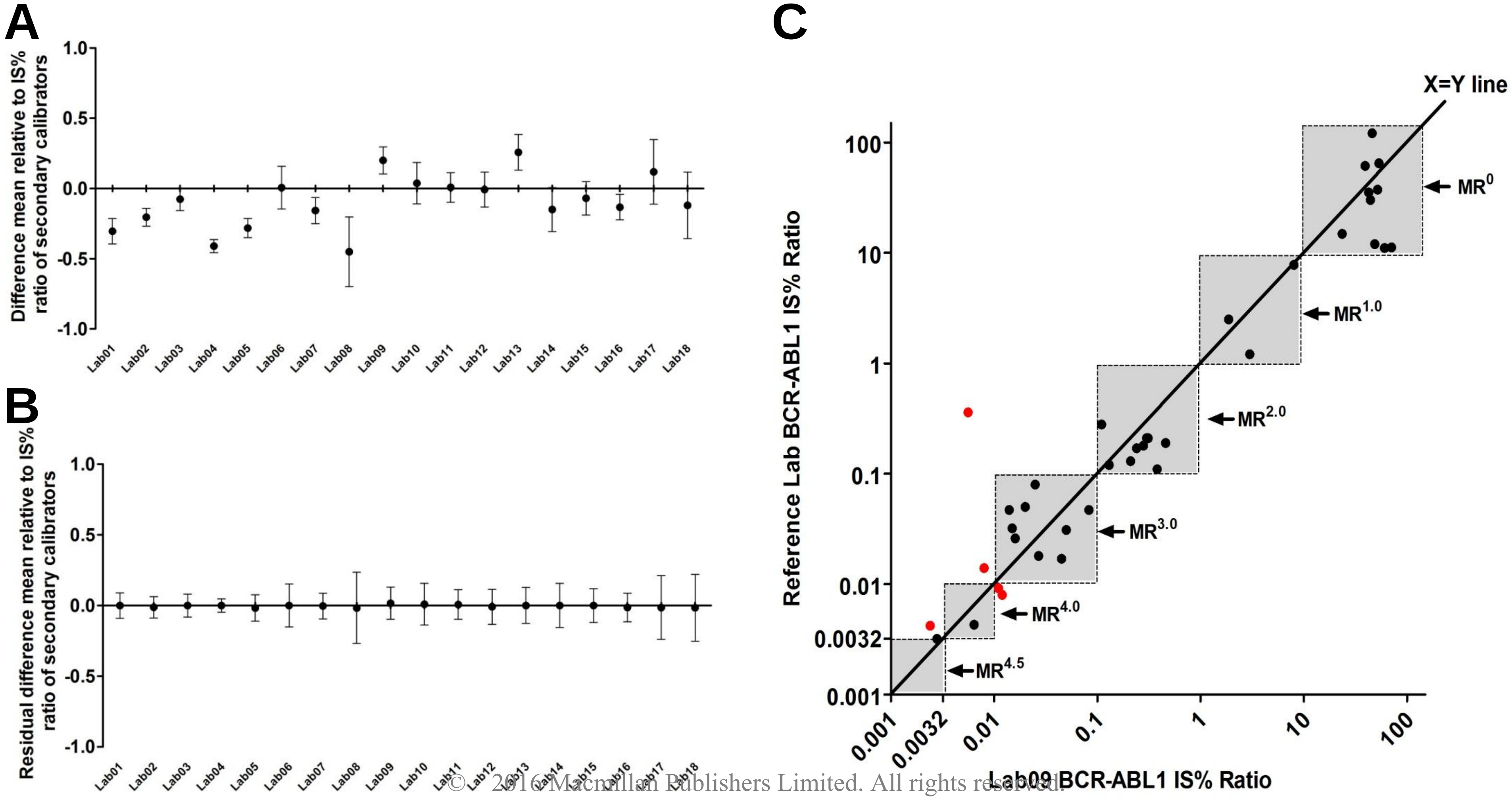

A
Difference mean relative to IS% ratio of secondary calibrators
1.0
0.5
0.0
-0.5
-1.0
Lab01
Lab02
Lab03
Lab04
Lab05
Lab06
Lab07
Lab08
Lab09
Lab10
Lab11
Lab12
Lab13
Lab14
Lab15
Lab16
Lab17
Lab18
B
Residual difference mean relative to IS% ratio of secondary calibrators
1.0
0.5
0.0
-0.5
-1.0
Lab01
Lab02
Lab03
Lab04
Lab05
Lab06
Lab07
Lab08
Lab09
Lab10
Lab11
Lab12
Lab13
Lab14
Lab15
Lab16
Lab17
Lab18
C
X=Y line
Reference Lab BCR-ABL1 IS% Ratio
100
10
1
0.1
0.01
0.0032
0.001
MR0
MR1.0
MR2.0
MR3.0
MR4.0
MR4.5
0.001
0.0032
0.01
0.1
1
10
100
Lab09 BCR-ABL1 IS% Ratio

Figure 2