An international study to standardize the detection and quantitation of BCR-ABL transcripts from stabilized peripheral blood preparations by quantitative RT-PCR

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ABSTRACT

Due to the lack of comparability of BCR-ABL mRNA quantification results generated by various methodologies in different laboratories, an international multicenter trial was started with the participation of six laboratories (platforms: LightCycler™, LC, n=3; TaqMan™, TM, n=3). One hundred and eighty-six PB samples derived from healthy donors were spiked with serial dilutions (1:20 to 1:2×10^6) of b2a2, b3a2 or e1a2 BCR-ABL positive white blood cells (WBC) from leukemic patients. After PAXgene™ stabilization, blinding, freezing and distribution, standardized RNA extraction, cDNA synthesis, PCR protocols and data evaluation were carried out. There was no significant difference in the results achieved using LC and TM technologies, but a considerable overall variation (CV=0.74 for ratios BCR-ABL/ABL). Up to a dilution of 1:1,000, 27/30 of the 2.5 mL samples tested positive. For higher dilutions, a PB volume of 5 or 10 ml was required to improve sensitivity. The study showed the feasibility of RQ-PCR standardization independent of the PCR machine used.

Key words: standardization, quantitative PCR, BCR-ABL, chronic myelogenous leukemia.

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Molecular monitoring by quantitative reverse transcriptase polymerase chain reaction (RQ-PCR) is a valuable technique in many fields of medicine. In contrast to microbiologic applications, such as the quantification of human immunodeficiency virus load,[1] there is a lack of standardized procedures and reference materials in leukemia surveillance. Because of the wide range of techniques used, it is difficult to compare results obtained from different laboratories. The most important variables between sample collection and final result are: (i) different volumes and cell counts of peripheral blood (PB) or bone marrow (BM) samples, different cell separation protocols such as the Ficoll™ procedure or red cell lysis; (ii) various transit times from blood collection until processing (with time-dependent RNA degradation);[2] (iii) different RNA extraction methods such as cesium chloride (CsCl) ultra centrifugation for high volumes of PB versus silica membrane based methods like the RNeasy Mini™ kit (Qiagen, Hilden, Germany) and PAXgene™ (PreAnalytiX, Hombrechtikon, Switzerland) with lower input capacity; (iv) heterogeneity of protocols for cDNA synthesis using specific or random hexamer priming and various RT enzymes; (v) different detection instrument platforms, e.g. TaqMan with hydrolysis probes, and LightCycler with hybridization probes; (vi) the use of different reference genes to compare BCR-ABL ratios to an internal control gene; (vii) different comparators for quantification, e.g. the comparison with serial plasmid dilutions or, alternatively, the use of an RNA calibrator as reference point for both target and reference genes. There are two ways to overcome the lack of comparability: a rigorous standardization of procedures or external calibration of results obtained with different methodologies. The first such standardization method has been applied in this

Table 1. Results of BCR-ABL/ABL and BCR-ABL/G6PD ratios separated by transcript type and dilution (d.l.: detection limit, representing samples being tested BCR-ABL positive only by a minority of laboratories).

Transcript	*Dilution*	*Volume of PB (mL)*	*BCR-ABL/ABL ratio*		*BCR-ABL/G6PD ratio*	
			median	*range*	*median*	*range*
b2a2	1:20	2.5	0.73	0.42-1.49	0.088	0.014-0.29
b2a2	1:50	2.5	0.38	0.19-0.89	0.047	0.0053-0.14
b2a2	1:100	2.5	0.20	0.077-0.40	0.032	0.0035-0.052
b2a2	1:1,000	2.5	0.026	0.0048-0.032	0.0025	0.00077-0.0066
b2a2	1:1,000	5.0	0.018	0.0023-0.038	0.0020	0.00065-0.0086
b2a2	1:1,000	10.0	0.012	0.0030-0.078	0.0016	0.00036-0.0097
b2a2	1:10,000	2.5	0.0024	0.00087-0.0037	0.00015	0.000042-0.00049
b2a2	1:10,000	5.0	0.0018	0.00020-0.0042	0.00016	0.000064-0.00031
b2a2	1:10,000	10.0	0.0017	0.000016-0.0045	0.00014	0.0000016-0.00041
b2a2	1:100,000	2.5	d.l.	d.l.	d.l.	d.l.
b2a2	1:100,000	5.0	d.l.	d.l.	d.l.	d.l.
b2a2	1:100,000	10.0	d.l.	d.l.	d.l.	d.l.
b3a2	1:20	2.5	0.31	0.025-0.42	0.057	0.021-0.086
b3a2	1:200	2.5	0.063	0.040-0.082	0.011	0.0013-0.014
b3a2	1:2,000	2.5	0.0032	0.0024-0.0092	0.00045	0.00016-0.00096
b3a2	1:20,000	10.0	0.00021	0.00000091-0.00092	0.000035	0.0000046-0.00012
b3a2	1:200,000	10.0	d.l.	d.l.	d.l.	d.l.
b3a2	1:2,000,000	10.0	d.l.	d.l.	d.l.	d.l.
e1a2	1:20	2.5	0.088	0.0087-0.25	0.0092	0.00017-0.025
e1a2	1:100	2.5	0.0084	0.00060-0.053	0.0015	0.000045-0.0048
e1a2	1:1,000	2.5	0.00092	0.000032-0.0051	0.000068	0.00000080-0.00093
e1a2	1:10,000	10.0	d.l.	d.l.	d.l.	d.l.
e1a2	1:100,000	10.0	d.l.	d.l.	d.l.	d.l.

trial, and the latter has been proposed by Hughes *et al.*[3] The focus of this study is on quantification of BCR-ABL mRNA transcripts. These levels have been shown to be a reliable surrogate parameter for progression free survival after treatment with interferon α or imatinib and after allogeneic stem cell transplantation[4-8] in patients with chronic myelogenous leukemia (CML). An international multicenter trial involving six laboratories from the United States of America (n=2), the United Kingdom (n=1), Italy (n=1), and Germany (n=2) was started with the aim of comparing results using a common procedure on two different platforms (LC and TM). We aimed to (i) demonstrate the feasibility of standardizing procedures; (ii) compare qualitative and quantitative MRD results from different platforms; (iii) determine the optimal PB volume for the clinically relevant detection of low tumor burden, and (iv) to confirm the applicability of total ABL and glucose-6-phosphate dehydrogenase (G6PD) as reference genes to calculate relative response levels.

Design and Methods

Patients and healthy controls

PB samples of two CML patients (67 year old male, b2a2=e13a2$^{BCR-ABL}$ transcript positive; 62 year old female, b3a2=e14a2$^{BCR-ABL}$ transcript positive) and one patient with acute lymphoblastic leukemia (ALL, 28 year old male, e1a2$^{BCR-ABL}$ transcript positive) with high tumor load (100% Ph positive metaphases in cytogenetics) were used for PB spiking from healthy donors (two male donors, age 29 and 30 years) after informed consent was obtained.

Sample preparation

PB samples of BCR-ABL positive patients were incubated with NH_4Cl solution to lyse red blood cells and washed in phosphate buffered saline. After manually counting the white blood cell (WBC) stock solutions with leukocyte concentrations corresponding to the healthy donors, WBC counts were prepared for each BCR-ABL transcript type. A total of 1,020 mL freshly drawn healthy donors' PB was used for the preparation of serial dilutions (1:20 to 1:2×10^6) using b2a2, b3a2 and e1a2 BCR-ABL positive WBC within three hours after phlebotomy. After including 108 negative controls without the addition of BCR-ABL positive cells, a total of 408 aliquots of 2.5 ml were injected into PAXgene™ tubes containing stabilizing agent, blinded and frozen at -20°C until shipment to the participating laboratories. Multiple samples of the same dilutions were prepared to compare sensitivity when using low (2.5 mL, one tube), intermediate (5 mL, two tubes), or high volume (10 mL, four tubes) PB (Table 1).

RNA extraction

After thawing, samples were incubated for two hours at room temperature and RNA extraction was performed in all laboratories following the protocol of the PAXgene Blood RNA Kit™. The concentrations of RNA eluates obtained from 2.5 ml PB (40 µL) were directly assessed photometrically and introduced to cDNA synthesis.

Pooling of samples (5 and 10 ml PB) required ethanol precipitation following standard protocols.

cDNA synthesis

RNA (35 µL, corresponding to a median amount of 4.5 µg in samples derived from one tube, 5.6 µg in samples derived from two tubes, and 15.3 µg in samples derived from four tubes) was transcribed into cDNA following a LightCycler t(9;22) Quantification Kit™ (Roche) protocol using random hexamer priming and AMV reverse transcriptase.

Quantitative real time RT-PCR on LightCycler platforms

In all samples (n=186; n=31 per center), quantitative real time reverse transcriptase polymerase chain reaction (RQ-PCR) for BCR-ABL, total ABL, and G6PD mRNA transcripts were performed in duplicates (186 sample PCR reactions and 96 control PCR reactions) using a modified LightCycler t(9;22) Quantification Kit™ and the LightCycler 1.0 apparatus (Roche). cDNA calibrator served as positive control for BCR-ABL, total ABL, and G6PD. This version of the Roche kit was supplemented by an ABL detection mix to compare ABL and G6PD within this study. PCR conditions conformed to the kit protocol.

Quantitative real time RT-PCR on TaqMan platforms

Primers, probes and PCR conditions employed on TaqMan platforms (ABI PRISM™ 7700) were used according to the Europe Against Cancer (EAC) protocol.[9] All samples (n=31) were examined in duplicates for BCR-ABL, total ABL, and G6PD (186 sample PCR reactions and 54 control PCR reactions).

Calculation of the results

Roche Relative Quantification Software™[10] was used centrally to determine BCR-ABL, total ABL, and G6PD transcript concentrations and to calculate the BCR-ABL/total ABL and BCR-ABL/G6PD ratios. The coefficient files, reflecting the amplification characteristics of the RNA calibrator provided with the Roche t(9;22) kit which might change from lot to lot, were taken for evaluating the LightCycler data. Coefficient files of the TaqMan data were calculated using TaqMan PCR for BCR-ABL, total ABL, and G6PD of dilution series of the calibrator RNA of the kit. Each BCR-ABL/control gene ratio was calculated from the mean of duplicate measurements of BCR-ABL and control genes.

Statistical methods

Correlation coefficients were calculated according to Spearman's rank test. Comparison of molecular results and RNA yields were performed using the non-parametric Mann-Whitney and Wilcoxon tests. All tests were calculated using the GraphPad Prism software (GraphPad Software, Inc., San Diego, CA, USA).

Results and Discussion

Efficiency of RNA extraction

After extraction, RNA concentration was measured by UV spectrophotometry using 5 µL out of 40 µL each. B2a2$^{BCR-ABL}$ samples showed median RNA concentrations of 136 µg/mL (n=48), 161 µg/mL (n=24), and 360 µg/mL (n=24) using one tube (2.5 mL PB), two tubes (5 ml PB), and four tubes (10 mL PB) respectively. B3a2$^{BCR-ABL}$ samples revealed 128 µg/mL (n=24) and 490 µg/mL (n=24) after RNA extraction of one or four tubes respectively. Median RNA concentrations in e1a2 samples were 110 µg/mL (n=24) and 531 µg/mL (n=18) using one or four tubes respectively. For all transcript types, four tube extraction revealed significantly higher RNA concentrations compared to one tube extraction (p<0.0001).

Correlation of BCR-ABL/ABL and BCR-ABL/G6PD ratios

After decoding and evaluating the raw data there was an excellent correlation between the BCR-ABL/ABL and BCR-ABL/G6PD ratios (r=0.96, p<0.0001, Figure 1A, *online supplement*) independent of the platform used. This agrees with previously reported clinical data.[11-13]

Correlation of BCR-ABL ratios in LC vs TM platforms

BCR-ABL/ABL and BCR-ABL/G6PD ratios correlated well comparing LC results with TM results (n=53, r=0.85, p<0.0001, Figure 1B, *online supplement*). and r=0.82, p<0.0001, respectively). Overall, mean TM ratios were approximately three times higher than LC ratios (p<0.0001) when using ABL and showed no difference when using G6PD as housekeeping gene (p=0.25). Differences in the amplification efficiency of target and control genes may be the reason for this. The introduction of laboratory-specific conversion factors adopted for an external calibrator could provide a solution.

BCR-ABL/ABL ratios: individual results of six laboratories

Individual results from six participating laboratories are shown in Figure 2A (*online supplement*) (b2a2$^{BCR-ABL}$), Figure 2B *online supplement*) (b3a2$^{BCR-ABL}$), and Figure 2C *online supplement*) (e1a2$^{BCR-ABL}$). Samples with low BCR-ABL burden showed a wider variation of results with inconsistent detection of BCR-ABL. The coefficients of variation (CV) for samples tested positive were comparable for BCR-ABL/ABL (CV=0.74) and BCR-ABL/G6PD ratios (CV=0.76). There is a linear relationship between the grade of dilution of the samples and the BCR-ABL/ABL ratios (r^2=0.9831, 0.9888 and 0.9909 for b2a2, b3a2 and e1a2 dilutions respectively).

Sensitivity and specificity

BCR-ABL was detected in 100 out of 138 positive samples evaluating BCR-ABL relative to ABL (72.5%) and in 96 out of 138 relative to G6PD (69.6%). None of the laboratories revealed the presence of BCR-ABL in the 48 blinded negative controls, indicating a 100% specificity. Overall sensitivity is difficult to interpret since patient tumor load clearly differed (median BCR-ABL/ABL ratio of the 1:20 dilutions were 0.73, 0.31 and 0.088 in b2a2, b3a2 and e1a2 samples respectively). Up to a dilution of 1:1,000, 27 out of 30 (90%) 2.5 mL samples tested positive. Two of the 3 failures derived from the 1:1,000 dilution of e1a2 samples containing lower BCR-ABL copy numbers than the respective b2a2 or b3a2 dilutions. For higher dilutions (1:10,000 to 1:2,000,000), an initial PB volume of 5 or 10 mL (2 tubes or 4 tubes) partly showed improved sensitivity (Figure 2A, *online supplement*).

We conclude that (i) standardization of quantitative PCR is feasible independent of the PCR machine used; (ii) test sensitivity depends on the volume of PB used. To detect a three-log reduction of the tumor load on a cellular basis (the clinical treatment target that best predicts optimal long-term survival), 2.5 mL PB seems to be sufficient in most cases. Higher sensitivities may be achieved using 10 mL PB; (iii) both total ABL and G6PD can be used as control genes. The comparability of RQ-PCR data does not depend on the use of identical platforms but on a common procedure to calculate results. External standards may help to control the differences in performance between laboratories.[3] International agreements on the use of a limited number of PCR protocols optimized for each platform are needed. The implementation of international control and calibrator materials, and the prospective use of these protocols within clinical trials would be advisable.

Authors' Contributions

MM, GS, LF: performed sample preparation, logistics, data evaluation, writing of the paper; HP: performed sample testing, contributed to writing of the paper; RP: performed sample testing, contributed to writing of the paper; RT: performed sample testing, contributed to writing of the pape; PP: sample preparation, logistics, performed sample testing, contributed to writing of the paper; EG: performed sample testing, contributed to writing of the paper; SB: logistics, contributed to writing of the paper; OO: logistics, contributed to writing of the paper; HS: logistics, contributed to writing of the paper; LW: logistics, contributed to writing of the paper; KM: performed sample testing, contributed to writing of the paper; HK: sample preparation, logistics, contributed to writing of the paper; US: sample preparation, contributed to writing of the paper; RH: contributed to writing of the paper; AH: logistics, writing of the paper.

Conflict of Interest

The author reported no potential conflicts of interest.

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