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Interlaboratory comparability – an important criterion for selection of a method for relative quantification of BCR-ABL

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SUMMARY
Objective: A BCR-ABL fusion transcript found in peripheral blood or bone marrow is a specific molecular marker of chronic myeloid leukemia and Philadelphia chromosome – positive acute lymphoblastic leukemia. Modern quantitative analyses in molecular biology are based on real-time PCR technology and fluorescent probes targeted to a complementary part of DNA/cDNA templates. A current trend in laboratory medicine and applied molecular biology is to support proficiency testing and transferability of results of quantitative PCR analyses. The goal of the study is to compare the diagnostic impact of two used systems for BCR-ABL quantification with respect to recommendations of the Europe Against Cancer Program (EAC) unifying the used BCR-ABL methodology.
Material and Methods: A total of 143 peripheral blood specimens and 5 bone marrow aspirates from 80 adult subjects were analysed. Two quantitative procedures were simultaneously examined. Procedure I was based on Superscript III reverse transcription (Invitrogen) and M-bcr FusionQuant Kit (Ipsogen) for relative quantification of BCR-ABL. In procedure II we used LightCycler t(9;22) Quantification Kit (Roche).
Results: The calibration curve for BCR-ABL gene (FusionQuant) was: CT = -3.54 . log(conc BCR-ABL) + 42.33; automatic threshold = 0.06, correlation coefficient (r) = -1.00, reaction efficiency = 92%. The Roche kit does not use any calibration process for BCR-ABL. The calibration curve for ABL gene (FusionQuant) was CT = -3.53 . log(conc ABL) + 40.91, ABL threshold = 0.08, r = -1.00, reaction efficiency = 91%. The calibration curve for G6PDH control gene (Roche) was CT = -3.2 . log(conc G6PDH) + 30.9, r = -1.00, reaction efficiency = 100%. The limit of quantification for BCR-ABL (FusionQuant) was 10 copies in 5 µl of the used cDNA. The lowest relative concentration of BCR-ABL detectable by the Roche assay was 0.001 %. The experimental data showed a close linear association in relative quantity of BCR-ABL received by the examined assays with r = 0.77, P < 0.001.
Conclusions: The principal difference between the tested procedures is the house-keeping gene used for normalization of BCR-ABL quantity. In agreement with the Europe Against Cancer strategy we have selected ABL gene as a normalization gene for our lab.
Key words: BCR-ABL, quantification, real-time PCR, house-keeping gene, ABL gene, EQA.

SOUHRN
Beránek M., Hegerová J., Voglová J., Bělohlávková P.: Mezilaboratorní porovnatelnost – důležité kritérium pro výběr metody pro relativní kvantifikaci BCR-ABL
Úvod: Fúzní transkript BCR-ABL, prokazatelný v periferní krvi nebo vzorku kostní dřeně, je specifickým molekulárním markerem chronické myeloidní leukémie a akutní lymfoblastické leukémie s nálezem Filadelfského chromozomu. Moderní kvantitativní molekulárně biologická vyšetření jsou založená na technologii real-time PCR a fluorescenčních sondách komplementárních vůči vyšetřovanému DNA/cDNA templátu. Současný trend v laboratorní medicíně a aplikované molekulární biologii podporuje snahu o externí hodnocení kvality a přenositelnost výsledků kvantitativních PCR analýz. Cílem studie je porovnat diagnostický význam dvou analytických systémů určených pro kvantifikaci BCR-ABL s ohledem na současná doporučení programu Europe Against Cancer Program (EAC).
Materiál a metody: Celkem bylo analyzováno 148 vzorků (143 vzorky periferní krve a 5 aspirátů kostní dřeně) získaných od 80 dospělých jedinců. Vzorky byly vyšetřeny paralelně dvěma kvantitativními postupy s normalizací na příslušný house-keeping gen. První postup byl založen na reverzní transkripci RNA pomocí enzymu Superscript III (Invitrogen) a kvantifikaci BCR-ABL soupravou M-bcr FusionQuant Kit (Ipsogen). Druhý postup používal soupravu LightCycler t(9;22) Quantification Kit (Roche).
Výsledky: Kalibrační rovnice pro BCR-ABL gen (FusionQuant) byla: CT = -3,54 . log(conc BCR-ABL) + 42,33; automatický threshold = 0,06, korelační koeficient r = -1,00, reakční účinnost = 92 %. Souprava firmy Roche nepoužívá pro kvantifikaci BCR-ABL genu kalibrační proces. Kalibrační rovnice pro ABL gen (FusionQuant) byla CT = -3,53 . log(conc ABL) + 40,91, threshold pro ABL = 0,08, r = -1,00, reakční účinnost = 91 %. Kalibrační rovnice pro G6PDH gen (Roche) byla CT = -3,2 . log(conc G6PDH) + 30,9, r = -1,00, reakční účinnost = 100 %. Minimální koncentrace BCR-ABL stanovitelná soupravou FusionQuant byla 10 kopií v 5 µl použité cDNA. Nejnižší relativní koncentrace BCR-ABL stanovitelná soupravou Roche byla 0,001 %. Experimentální data ukázala lineární závislost mezi hodnotami relativní koncentrace BCR-ABL dosaženými oběma metodami s korelačním koeficientem r = 0,77, p < 0,001.
Závěr: Mezi principiální rozdíly obou testovaných souprav patří výběr house-keeping genu použitého pro normalizaci koncentrace BCR-ABL. V souladu s doporučeními programu Europe Against Cancer jsme pro rutinní vyšetření v naší laboratoři zvolili soupravu FusionQuant s normalizací na ABL gen.
Klíčová slova: BCR-ABL, kvantifikace, real-time PCR, house-keeping gen, ABL gen, EQA.

Introduction

A BCR-ABL fusion transcript found in peripheral blood or bone marrow is a specific molecular marker of chronic myeloid leukemia (CML) and Philadelphia chromosome-positive (Ph^+) acute lymphoblastic leukemia (ALL). The number of BCR-ABL transcripts reflects the current clinical status of the patient and in many cases provides very important predictive information. In other words, molecular relapse confirmed by a significant elevation of BCR-ABL expression in the biological material portends a worse cytogenetic, hematological, and clinical response to the therapy or a lower donor graft activity after allogeneic transplantation of peripheral blood stem cells.

The accurate amount of measured BCR-ABL transcripts and its correct clinical interpretation depend on the used analytical method. Modern quantitative analyses in molecular biology are based on real-time PCR technology and fluorescent probes targeted to a complementary part of DNA/cDNA templates. The number of BCR-ABL transcripts in the biological material is determined from the calibration curve constructed using calibration standards with known concentrations of BCR-ABL molecules. Absolute quantification in molecular biology is, however, negatively influenced by the integrity of RNA molecules in clinical specimens, and efficiency of reverse transcription (RT) and polymerase chain reaction (PCR). Taking into account all these factors, relative quantification based on internal normalization of BCR-ABL numbers to the expression of house-keeping genes (HKG) in the same specimen, is generally preferred.

A lot of house-keeping genes (ABL, BCR, β-glucuronidase /GUS/, β2-microglobulin /B2M/, glucose-6--phosphate dehydrogenase /G6PDH/, glyceraldehyde-3--phosphate dehydrogenase /GAPDH/, TATA-box binding protein /TBP/, 18S rRNA, β-actin) for BCR-ABL normalization was described previously [1–4]. However, different expression of HKG resulting in a different BCR-ABL relative quantity could lead to clinical misinterpretations and influence the sensitivity of the used method.

A current trend in laboratory medicine and applied molecular biology is to support proficiency testing and transferability of results of quantitative PCR analyses. This trend determines development of a new generation of diagnostic kits, reference materials, laboratory equipment, and also the choice of appropriate house--keeping genes.

The goal of the study is to compare the diagnostic impact of two systems used for BCR-ABL quantification with respect to recommendations of the Europe Against Cancer Program (EAC) unifying the used BCR-ABL methodology, and improving interlaboratory comparability and collaboration in Europe [1, 5, 6].

Materials and Methods

Specimens. A total of 143 peripheral blood specimens and 5 bone marrow aspirates from 80 adult subjects (40 men and 40 women in a median age of 57 years, range 22-86 years) were analysed. The specimens were received at the time of diagnosis (twenty specimens) and the reminder was obtained during regular follow-up visits. The specimens originated from 65 CML patients (26 b2a2 and 39 b3a2 major types), 8 ALL patients with BCR-ABL minor type (e1a2), and 7 subjects suffering from myeloproliferative syndromes with no evidence of a Ph chromosome. The BCR-ABL analysis was performed with informed consent of patients.

Leukocytes separation. Bone marrow (0.2–2.0 ml) or blood specimens (5–7 ml) were processed maximally to 2 hours after collection. Leukocytes were separated by red cells hypotonic lysis, washed in phosphate-buffered saline and counted.

Total RNA isolation. Total leukocyte RNA from 10^7 cells per sample was prepared from cell lysates according to a modified Chomczynski and Sacchi extraction method [7–8]. RNA concentrations were determined by absorbance measurements at 260 nm. Usually 5–30 µg of RNA in 30 µl was recovered. After extraction, RNA was immediately used for cDNA synthesis, and the rest stored at -80 °C.

First-strand cDNA synthesis and real-time PCR. Two quantitative procedures were simultaneously tested:

Procedure I. Five hundred nanograms of RNA were heated to 70 °C for 10 min and immediately placed on ice. The final reaction mixture contained 10 µl of 2x concentrated RT Reaction Mix (includes 2.5 µM oligo$(dT)_{20}$, 2.5 ng/µl random hexamers, 10 mM $MgCl_2$ and dNTPs), 2 µl of RT Enzyme Mix (includes SuperScript III RT and RNaseOUT), heat-denaturated RNA and DEPC-treated water to 20 µl. Synthesis of cDNA was achieved by 10 min incubation at 25 °C, 50 min incubation at 50 °C and terminated by heating at 85 °C for 5 min. After that, 1 µl (2 U) of *E. coli* RNase H was added to the mixture and the process was finished by 20 min incubation at 37 °C according to the instructions of manufacturer (Invitrogen, Carlsbad, USA). For quantification of BCR-ABL transcripts we used M-bcr FusionQuant Kit (Ipsogen, Marseille, France). The kit serves for *in vitro* diagnostic use (IVD) and detects major BCR-ABL translocations, M-bcr (b3a2, b2a2, and also rare cases b2a3 and b3a3). 5 µl of cDNA were added to the master mix composed from 12.5 µl of 2x concentrated TaqMan Universal PCR Master (Applied Biosystems, manufactured by Roche, Branchburg, New Jersey, USA), 1 µl of 25x concentrated Ipsogen Primers and Probe mix (FusionQuant Kit), and 6.5 µl of nuclease-free water. Real-time PCR amplification was performed in RotorGene 6000 (Corbett Research, Mortlake, Australia). Thermocycling and fluorescence acquisition conditions were set according to the recommendations of the manufacturer. The relative number of BCR-ABL transcripts in the samples was expressed in percentages after normalization to ABL house-keeping gene. Calibration curves for BCR-ABL and ABL were constructed using a series of cDNA calibration standards containing 10^1–10^6 copies in 5 µl of cDNA, and 10^3–10^5 copies respectivelly (FusionQuant, Ipsogen).

Procedure II. Reverse transcription and real-time PCR were carried out in the LightCycler t(9;22) Quantification Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's recommendations. The kit is able to detect major (b2a2, b3a2, b2a3, b3a3) and minor (e1a2) translocations. Up to 1 µg of total RNA was usually taken per one reaction. 5 µl of cDNA were added to the master mix prepared according to the original manufacturer´ s protocol. PCR was performed in the LightCycler Instrument (Roche). Calibration curves were constructed using three standards containing purified transcripts of the glucose 6-phosphate dehydrogenase (G6PDH) gene. The number of BCR--ABL transcripts in clinical samples was normalized in ratio to the amount of G6PDH RNA transcripts.

Statistical analysis. Duplicate PCR reactions were performed for each specimen, calibration standard, and negative control sample. Results were expressed as mean values. The equations of standard curves were calculated by least-squares analysis. Precision of the procedure was expressed as a coefficient of inter--assay variation (CV) and provided in percentages. The mean values were analysed by correlation and linear regression analysis. P-values in a *t* test < 0.05 were considered statistically significant.

Results and Discussion

A calibration process used in quantitative real-time PCR reflects the linear dependency of the number of cycles necessary to detect a first positive fluorescent signal above a threshold of detection (CT, cycle threshold) on the decadic logarithm of the DNA concentration in the sample. For construction of BCR-ABL and ABL calibration curves, the Fusion-Quant kit uses the series of calibration standards with known concentrations of cDNA. The standards were prepared by recombinant plasmid technology. Five standards for BCR-ABL gene calibration contained 10^1, 10^2, 10^3, 10^5, and 10^6 copies in 5 µl of cDNA; three ABL standards contained 10^3, 10^4, and 10^5 copies. Each calibration point on the curve represents the average value received from two analyses of the calibrator performed in the same run. The calibration curve for BCR-ABL gene was:

CT = -3.54 . log(conc BCR-ABL) + 42.33 (Fig. 1 and 2); automatic threshold = 0.06, correlation coefficient (r) = -1.00, reaction efficiency = 92%, and coefficients of variation (CV) for BCR-ABL standards varied from 0.8 to 14.1%. The Roche kit does not use any calibration process for BCR-ABL.

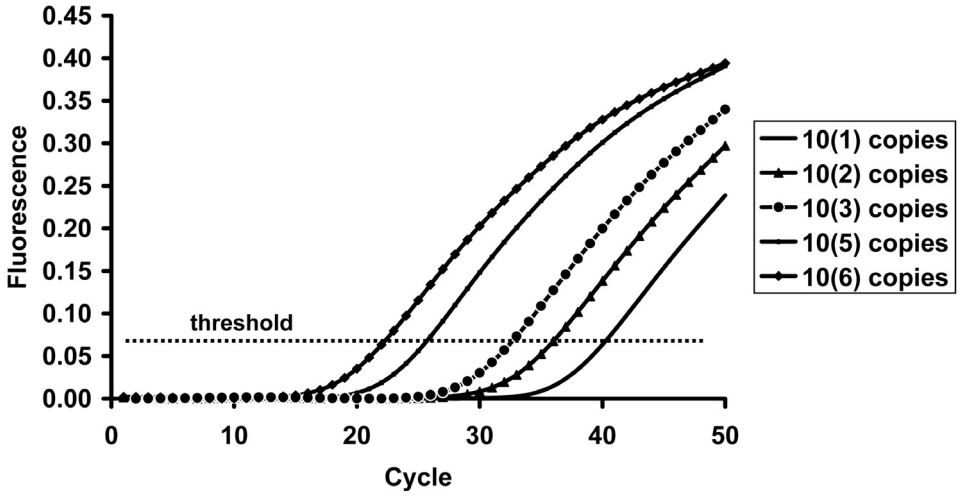

Fig. 1. Amplification curves of cDNA calibration standards for BCR-ABL quantification

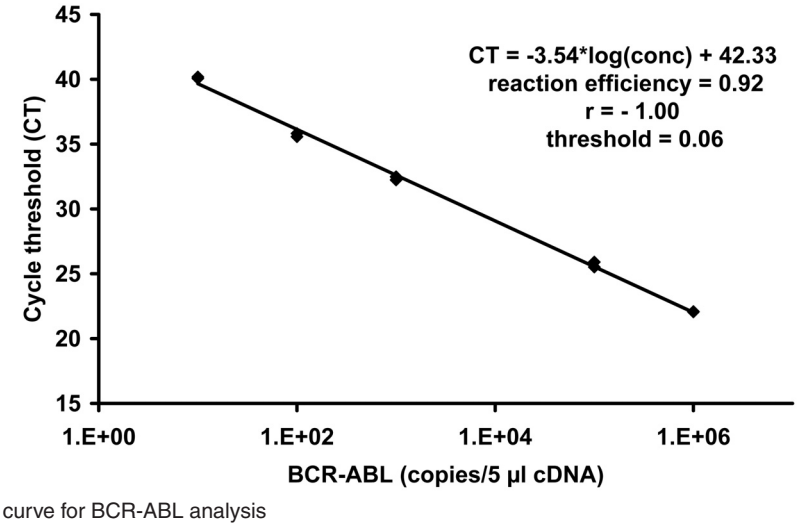

Fig. 2. Calibration curve for BCR-ABL analysis

The quantity of BCR-ABL measured by the FusionQuant kit is normalized to ABL gene. The calibration curve for ABL gene was CT = -3.53 . log(conc ABL) + 40.91, ABL threshold = 0.08, r = -1.00, reaction efficiency = 91%, and CV 3.5–6.3%. The calibration curve for G6PDH control gene in the Roche kit was CT = -3.2 . log(conc G6PDH) + 30.9, r = -1.00, reaction efficiency = 100%. CVs for the calibration standards were ≤ 10 %.

The manufacturer of the FusioQuant kit has not directly mentioned the limit of quantification (LOQ) and dynamic measurement range for BCR-ABL and ABL genes in the instructions for users. Therefore, the sensitivity and measurement range for BCR-ABL and HKG were verified by a dilution experiment using the calibration standard with the highest cDNA concentration. Each serial dilution with RNase-free water reduced the amount of cDNA in the defined volume by a factor of ten. The determined LOQ for BCR-ABL and ABL genes (FusionQuant) was 10 copies in 5 µl of cDNA. Our data are in agreement with results of other studies carried out in ABI Prism 7700 (Applied Biosystems) and LightCycler instruments [9–12].

In the FusionQuant analysis a positive signal of increasing fluorescence above the threshold was obtained also in blood specimens containing less than 10 copies of BCR-ABL. In some cases, however, an amplification curve appeared just in one of two simultaneously examined tubes. Such results could not be precisely quantified. Taking into account the range of BCR-ABL copies in the calibration standards (10^1–10^6), lower numbers of BCR-ABL in the clinical sample should be interpretated as "*lower than LOQ*".

Our dilution experiment verified that the linear range of measurement for both BCR-ABL and ABL genes is from 10^1 to 10^6 copies, or 10^5 copies respectivelly, if the pure calibration standard containing one type of cDNA (BCR-ABL or ABL) is examined. In cDNA samples received from real biological material BCR-ABL and ABL products are co-amplified because of the same primer type used for elongation of ABL gene side. This phenomenom reduces the range of linearity of relative quantitation from 0.01% to 10%, if ABL normalization has been used. Results of the samples containing more than 10% of fusion transcripts are underestimated in comparison with other HKG normalization [13, 14].

Thus, the principal difference between the tested procedures is the choice of house-keeping gene used for normalization of BCR-ABL quantity. The Roche kit uses G6PDH RNA calibrators with defined concentrations from the range ~0.5–1500 fg. Since the molecular weight of G6PDH RNA molecules is not provided, any estimation of the number of copies is not possible. Therefore, we expressed the sensitivity of the Roche quantitative procedure after G6PDH normalization. The lowest detectable relative concentration of BCR-ABL is 0.001 % [15].

Beillard et al. considered ABL values within the range $1.3x10^3$ – $1.3x10^5$ copies to be amplifiable and qualified for subsequent evaluation [1]. From this reason, three cDNA samples with ABL expression lower than 1300 copies (443, 761, and 816 copies) were excluded from further analysis. Absolute quantities of ABL gene transcripts in the evauated specimens fluctuated between 1800 and 18450 copies/5 µl cDNA (median 2719 copies) according to the RNA quality. The CV values in all duplicate PCR reactions for ABL gene were lower than 25% with an average value of 5.41%.

Inter-assay variability was evaluated on 43 FusionQuant BCR-ABL positive specimens. The concentration of BCR-ABL fusion transcripts varied in them from 2.7 to ~10^4 copies/5 µl cDNA. The mean inter-assay CV for BCR-ABL achieved 19.19%. The CV values increased with decreasing BCR-ABL quantity in the specimens (Fig. 3). Seven from the examined samples with BCR-ABL quantity lower than 30 copies revealed a coefficient of variation higher than 40 %. Our previous validation study on the Roche assay (15) and other studies found a similar inter-assay precision (9, 16). In three of the seven samples the mean value of BCR-ABL quantity was lower than LOG; in four remaining samples the mean values moved between 12 and 30 copies/5 µl cDNA. Such inter-assay variability in this part of measurement range is not considered clinically significant.

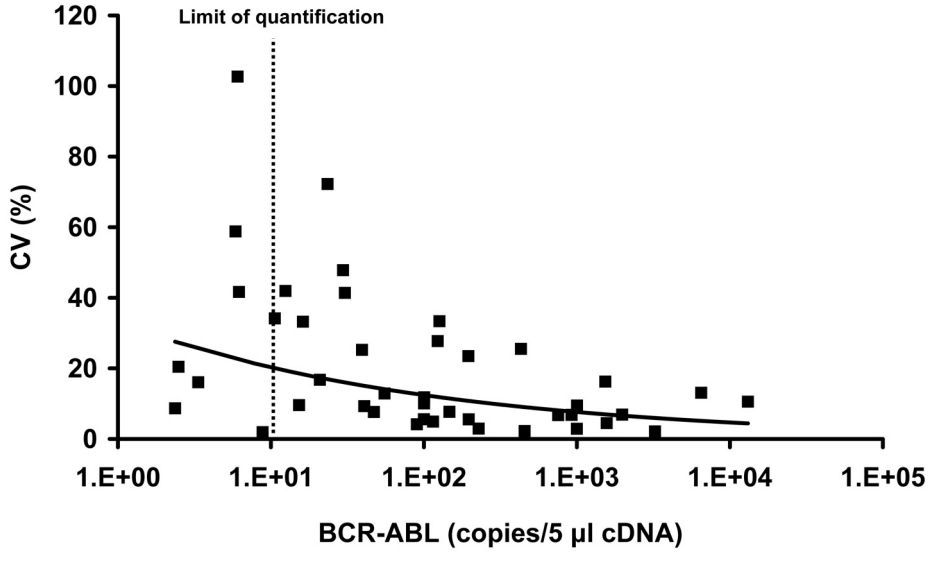

Fig. 3. Coefficients of inter-assay variation (CV) related to the BCR-ABL concentration

A following part of the study compared FusionQuant results with the data received by the Roche kit which has been using in our lab since 1999. RNA transcripts extracted from the same clinical specimens were processed simultaneously by both real-time procedures. BCR--ABL positivity detected by both the methods was found in sixty cases. The relationship between the pair results showed a close linear association in relative quantity of BCR-ABL with a correlation coefficient (r) of 0.77;

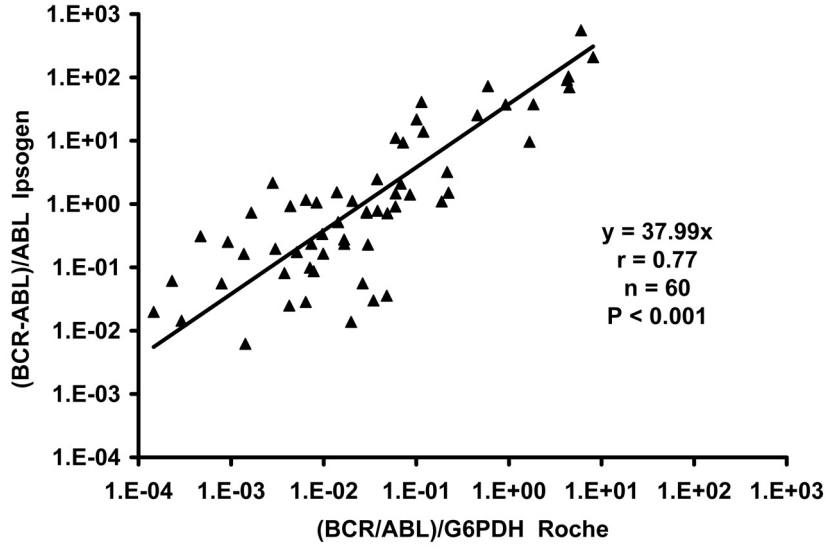

Fig. 4. Comparison of the relative quantity of BCR-ABL received by the Ipsogen (y-axis) and Roche (x-axis) quantification kits

y = 37.99x; P < 0.001; degree of freedom (df) = 59. Fig. 4 shows the comparability of the assays used for BCR--ABL quantification after normalization to the appropriate house-keeping gene in a log-log scale design. A weaker association between absolute BCR-ABL quantities (r = 0.58; y = 68.68x; P < 0.005) was also observed. On the contrary, we did not find a significant relationship between expression of ABL and G6PDH house-keeping-genes (r = 0.18; y = 9.77x, NS) in the examined specimens.

Twenty-six other specimens with b3a2 (n = 14) or b2a2 (n = 12) fusion type provided a positive BCR--ABL signal only if the FusionQuant kit was used. The data indicate a higher sensitivity of that assay in comparison with the Roche one. All eight specimens originated from Ph^+ ALL patients with the minor BCR--ABL translocation (= e1a2 type) gave positive results in the Roche procedure only. The FusionQuant kit is not determined for detection of BCR-ABL minor transcripts and this experiment confirmed its 100% analytical specificity for b3a2 and b2a2 transcripts. Fully concordant BCR-ABL negative results were observed in the group of specimens collected from the patients with other diagnosis than CML or Ph^+ ALL (n = 13).

The rest of the examined specimens (n = 38) provided BCR-ABL negative results. All of them were collected and examined during regular follow-up of succesfully treated CML patients. The concentrations of BCR-ABL in them were lower than detection limits of both the used procedures. Analytical data are summarized in Table1.

A contemporary harmonizing activity of EAC has unified the most suitable house-keeping genes. The common criteria for selection of an optimal HKG include: i) absence of pseudogenes, ii) expression level comparable with BCR-ABL at diagnosis, iii) no significantly different expression between normal and leukemic peripheral blood samples, iv) similar expression in bone marrow and blood, v) stability similar to BCR-ABL during the storage, vi) absence of alternative transcritional start sites, vii) absence of genetic lesions impairing the primer/probe annealing, viii) cell-cycle independent expression, and ix) gene location outside the X-chromosome [1, 3, 4]. According to EAC protocols, the most labs in the world have used ABL, B2M or GUS normalization. ABL gene, most frequently used HKG at all, has three disadvantages: 1) BCR-ABL/ABL relative quantity calculation could reach maximally 100 % leading to misinterpretation in untreated Ph^+ ALL patients and CML patients in advanced phases, 2) the use of the same ABL gene side primer for BCR--ABL and ABL amplification influences the results for both the genes during the therapy, and 3) ABL gene expression could be upregulated in response to cellular stress [4, 5].

Taking into account the heterogeneity of BCR-ABL relative quantification performed using very stable and sensitive biotechnologies, conversion of local laboratory values to an international scale has been proposed to allow alignment of data and reduce the interlaboratory variability [3, 6]. The effective measurement range for the international scale was deemed to be a BCR-ABL level of 10% or bellow [13]. Reference laboratory for BCR-ABL, Institute of Hematology and Blood Transfusion, Prague (web site: http://www.uhkt.cz) started a national program on BCR-ABL quantity in 2007. Similarly like other external quality assessment (EQA) systems for molecular biology [17], the coordinators provide reference samples, evaluate submitted results, compare them with the reference ones, and also calculate interlaboratory conversion coefficients

Table 1. Characteristics and experimental data on examined BCR-ABL procedures

PROCEDURE	I.	II.
Reverse transcription (RT)	Invitrogen	Roche
RNA in RT	500 ng	up to 1 µg
RT volume	20 µl	20 µl
RT enzyme	Superscript III	AMV
RT temperature	37 °C	50 °C
cDNA volume/PCR	5 µl	5 µl
BCR-ABL quantity	Ipsogen	Roche
Calibrators provided	yes	no
Calibration range	10^1–10^6	–
BCR-ABL units	copies/5 µl cDNA	fg of G6PDH RNA
BCR-ABL results range	2.8–49640	0.003–772
BCR-ABL median	168.3	0.36
HKG quantity	Ipsogen	Roche
HKG used	ABL	G6PDH
Calibrators provided	yes	yes
Calibration range	10^3–10^5	1–10^3
HKG units	copies/5 µl cDNA	fg of G6PDH RNA
HKG results range	1800–18450	52–13020
HKG median	2719	1244
Relative quantity BCR-ABL		
RQ BCR-ABL range (%)	0.006–554	0.0001–8.11
RQ BCR-ABL median (%)	0.78	0.03
Turnaround time	3 days	3 days
Real time of analysis	9 hours	7 hours

for EQA participants. Our conversion coefficient for the Roche and FusionQuant assays were 3.57 (in 2007) and 1.02 (in 2008) respectivelly.

Thus, despite using different analytical systems and house-keeping genes, the conversion coefficient is a first objective tool how to i) systematically adjust patient data received from various health centres, ii) standardize laboratory and medical care for CML patients in our country, and iii) unify the format of results (and units) in world medical databases.

Conclusions

Quantitative real-time PCR for BCR-ABL fusion transcripts is a very important laboratory method for evaluation of effectivity of CML and Ph^+ ALL treatment. Our study compared the diagnostic impact of two commercial systems for BCR-ABL quantification. Both systems provided reliable and reproducible results for BCR-ABL within a wide dynamic range. In agreement with the Europe Against Cancer strategy we have selected ABL gene as a normalization gene for our lab.

LightCycler t(9;22) Quantification Kit using G6PDH normalization is able to detect major and minor types of BCR-ABL translocation. The kit contains the reagents for reverse transcription and real-time PCR but not calibration standards for BCR-ABL. It is not possible to express absolute quantity of BCR-ABL in copy numbers. G6PDH gene does not belong to recommended HKGs for BCR-ABL analysis.

M-bcr FusionQuant Kit has been designed according to EAC studies and uses ABL normalization. The kit is certified for IVD and provides calibration standards and reagents for BCR-ABL real-time PCR (for BCR--ABL major type only). The standards are prepared as cDNA templates with defined copy numbers. The system does not contain reagents for reverse transcription and control RNA samples. The FusionQuant kit provided more sensitive results in comparison with LightCycler t(9;22) Quantification Kit.

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