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LightCycler Technology for the Quantitation of bcr/abl Fusion Transcripts

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ABSTRACT

Quantifying bcr/abl fusion transcripts in chronic myelogenous leukemia is thought to serve as a powerful parameter for monitoring the kinetic nature of this clonal disease *in vivo* and *in vitro*. Recently, we demonstrated the technical advantages as well as the clinical relevance of quantitating bcr/abl fusion mRNA using the 5-nuclease assay and a real-time fluorescence reverse transcriptase-PCR (RT-PCR) detection system (ABI PRISM 7700 SDS). Meanwhile, another technique was introduced (LightCycler technology) that may be used for the same purpose. To investigate whether this method may be an appropriate alternative to the described procedure, we have established bcr/abl LightCycler RT-PCR for major and minor bcr/abl fusion transcripts. We found that, with only minor modifications, TaqMan RT-PCR and fluorescent probe design can be used to obtain comparable results in the LightCycler system. The developed method could quantitate as little as 10 bcr/abl copies per 100 ng cDNA and was as safe and reproducible as the previously described technique. Because reaction efficiency was identical within different bcr/abl major fusions, one single RT-PCR could be established that simultaneously detects b2a3, b2a2, b3a2, and b3a3 fusion RNA with equal specificity and sensitivity. Compared to results generated by the ABI PRISM 7700 SDS, absolute amounts of bcr/abl did not differ significantly, and there was a linear correlation between the respective values. We conclude that TaqMan chemistry can be used in the LightCycler and that both real-time fluorescence PCR detection systems equally fulfill the criteria for the safe and reliable quantitation of bcr/abl fusion RNA in clinical samples. This may be of help for further standardization of quantitative bcr/abl RT-PCR, which, again, is necessary for the comparison of results generated by different investigators.

INTRODUCTION

The Philadelphia chromosome, resulting from the genetic rearrangement t(9;22)(q34;q11), is a hallmark of CML[2] (1, 2). The resulting fusion RNA encodes for a M_r 210,000 or 190,000 protein, which is thought to initiate malignant transformation of normal hematopoetic stem cells through enhanced activation of intracellular tyrosine kinases (3, 4). Because CML is a clonal disease, theoretically, detection of bcr/abl fusion proteins ($p210^{bcr/abl}$ and $p190^{bcr/abl}$) should precisely reflect CML disease activity; however, thus far, attempts focusing on the protein level did not reach the necessary specificity and sensitivity to be applied in clinical procedures (5, 6). Hence, compared to other approaches, molecular techniques are considered to be superior for the disease monitoring of CML patients. Qualitative PCR detection of bcr/abl fusions is well established in CML diagnostics, and PCR positivity virtually proves this type of leukemia (7, 8). On the basis of results obtained by semiquantitative PCR techniques, it was suggested that increasing numbers of bcr/abl fusion transcripts may directly reflect the expansion of a bcr/abl-positive clone (9), and conversely, it was speculated that decreasing bcr/abl RNA may be indicative of sufficient therapy (10, 11). Indeed, we and others have found that with a novel real-time fluorescence PCR detection system bcr/abl quantitation in peripheral blood cells is quick and easy to perform and provides meaningful parameters for the molecular monitoring in CML patients.[3] This assay bases on a target-specific probe labeled with fluorescent reporter and quencher dyes at it opposite ends (12). The probe is hydrolyzed through the 5′-nuclease activity of Taq DNA polymerase, leading to an increasing fluorescence emission of the reporter dye that can be detected during the reaction.

Recently, a new on-line fluorescence PCR detection system was introduced (LightCycler; Ref. 13). Originally, the so-called HybProbe chemistry was developed to be used in the LightCycler system. In contrast to TaqMan chemistry, this probe format uses two oligonucleotides, one labeled with a fluorescent dye at the 3′ terminus and the other carrying a dye at the 5′ end. The probes are designed to hybridize to the target strand, so that both dyes are in close proximity. In this system, one dye acts as donor fluorophore, whereas the other (acceptor) emits light if it is positioned near the donor dye. Typically, a fluorescein derivative is used as a donor, whereas a rhodamine or cyanine derivative acts as an acceptor. Thus, using this probe, chemistry in the LightCycler acceptor fluorescence emission is measured during the annealing step when both probes hybridize to the target strand. Analogous to the ABI PRISM 7700 SDS method, fluorescence is measured during PCR. It is suggested that the HybProbe format is more sensitive as measurable fluorescence is not dependent on the 5′-nuclease activity of the *Thermophilus aquaticus* DNA polymerase, whereas the acceptor fluorescence emission is maximized by a very close distance between both dyes. Here, we show that, with a modified protocol, TaqMan chemistry can be easily transferred to the LightCycler, leading to comparable results in both systems. This information may be useful for those who intend to use this alternative technology for *in vivo* or *in vitro* diagnostics.

MATERIALS AND METHODS

Standard Preparation. b2a2, b2a3, and b3a3 fusion transcripts were amplified in RT-PCR and cloned from patients known to carry the respective breakpoint. b3a2 and e1a2 clones were prepared from K562 cells (TOPO TA Cloning Kit; Invitrogen, Leek, The Netherlands). Cloned products were digested with *Hin*dIII and *Xba*I (Boehringer Mannheim, Mannheim, Germany), extracted from 3% agarose gel, and reamplified. Finally, the products were purified (PCR Purification Kit; Qiagen, Hilden, Germany) and measured in a photometer, and molecule concentrations were calculated. Serial dilutions ranging from 10^7 to 10^{-2} molecules per 100 ng of DNA [in herring sperm DNA and Tris-EDTA buffer (pH 8.0)] were then prepared.

Samples. Blood samples were collected into sterile heparin-containing tubes, separated by dextran sedimentation, and lysed. Total RNA from all samples was then extracted by a guanidinium isothiocyanate-acid phenol procedure (14), reverse-transcribed into cDNA with random hexamer primers, and stored at −20°C until assay.

RT-PCR. PCR of bcr/abl was performed using a single pair of primers located in exon 1 (m-bcr/abl, 5′-AGATCTGGCCCAACGATGG) or exon 2

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[2] The abbreviations used are: CML, chronic myelogenous leukemia; RT-PCR, reverse transcriptase-PCR; M- and m-bcr/abl, major and minor bcr/abl, respectively; nt, nucleotide(s); ROX, 5,6-carboxy-*x*-rhodamine.

[3] K. A. Kreuzer, U. Lass, S. Nagel, H. Ellerbrok, G. Pauli, B. Pawlaczyk-Peter, W. Siegert, D. Huhn, and C. A. Schmidt. A rapid and sensitive method for the absolute quantitation of bcr/abl fusion transcripts in chronic myelogenous leukemia, submitted for publication.

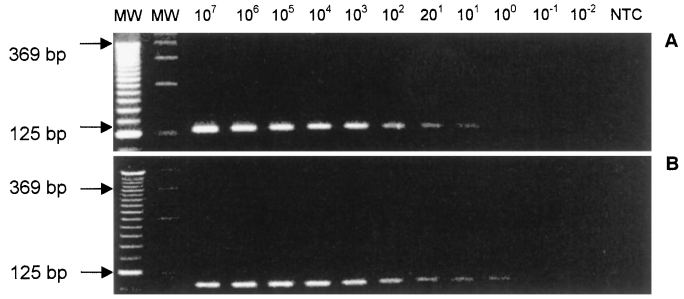

Fig. 1. M-bcr/abl (*A*) and m-bcr/abl (*B*) standard series after 45 cycles of conventional PCR amplification. Shown are 3% agarose gels after electrophoresis and ethidium bromide staining. Sizes are: M-bcr/abl (b2a3 fusion), 127 bp; m-bcr/abl (e1a2 fusion), 109 bp. *Top*, absolute numbers of starting templates; *NTC*, nontemplate control.

(M-bcr/abl, 5′-AGCATTCCGCTGACCATCA) of *bcr* and in exon 2 (m-bcr/abl, 5′-AGCGGCTTCACTCAGACCC) or exon 3 (M-bcr/abl, 5′-GCGTGATGTAGTTGCTTGGGAC) of *abl*. The fluorescent probes (M-bcr/abl, 5′-TTTGGGCTTCACACCATTCCCCATTG; m-bcr/abl, 5′-AGGCTCAAAGTCAGATGCTACTGGCCG) were designed to hybridize to the antisense strand of *abl*. Probes were labeled with 6-carboxy-fluorescein phosphoramidite at the 5′ end, and as a quencher, 5-carboxy-tetramethyl-rhodamine was incorporated at nt 9 (M-bcr/abl) or at nt 19 (m-bcr/abl) of the probe sequence (TIB Molbiol, Berlin, Germany). To prevent probe extension, phosphate groups were attached to the 3′ ends. The 50-μl PCR reaction mix contained 5 μl of 10× PCR buffer, 4.5 mM $MgCl_2$, 0.8 mM dNTP (Life Technologies, Inc., Karlsruhe, Germany), 1 μM ROX, 0.5 μM each primer, 1 μM probe, 1.25 units of a temperature-release Taq DNA polymerase (Platinum DNA polymerase; Life Technologies, Inc.), and 100 ng of sample cDNA. For LightCycler PCR, no ROX was used, but 30 μg of BSA were added to a 20-μl reaction mix. In conventional PCR probe, ROX and BSA were omitted. PCR amplification began with a 5-min denaturation step at 94°C, followed by 45 cycles of denaturation at 94°C for 30 s and annealing/extension at 65°C for 60 s. All experiments were performed in quintuplicate.

Data Analysis. Statistics were performed using Excel computer software (Microsoft Inc., Redmond, WA). *P*s < 0.01 were considered to be significant.

RESULTS

Fig. 1 shows ethidium bromide staining of conventional agarose gel electrophoresis after amplification of the b3a2 and e1a2 bcr/abl fusion standard series. With this approach, positive results after 45 cycles were obtained for M-bcr/abl and m-bcr/abl ($10–10^7$ copies per 100 ng of cDNA).

In Fig. 2, LightCycler fluorescence detection during PCR of the four M-bcr/abl fusions and the m-bcr/abl transcript is shown. Here again, all fusions were detectable until the target number was <10 copies per 100 ng of cDNA.

Quantitative results of real-time fluorescence PCR are assessed by determination of the cycle threshold value (*Ct*), which marks the cycle when fluorescence of a given sample becomes significantly different from the baseline signal. In the ABI PRISM 7700 SDS method, this background fluorescence is calculated automatically and is defined by the 10-fold SD of background fluorescence of all samples during the first 15 PCR cycles. Because LightCycler analytical software does not provide such an algorithm, we calculated LightCycler *Ct*s using the ABI PRISM 7700 SDS algorithm. Fig. 3 illustrates calibration curves obtained by plotting the initial number of target molecules in the standard series against the respective *Ct*s. For all bcr/abl RT-PCRs,

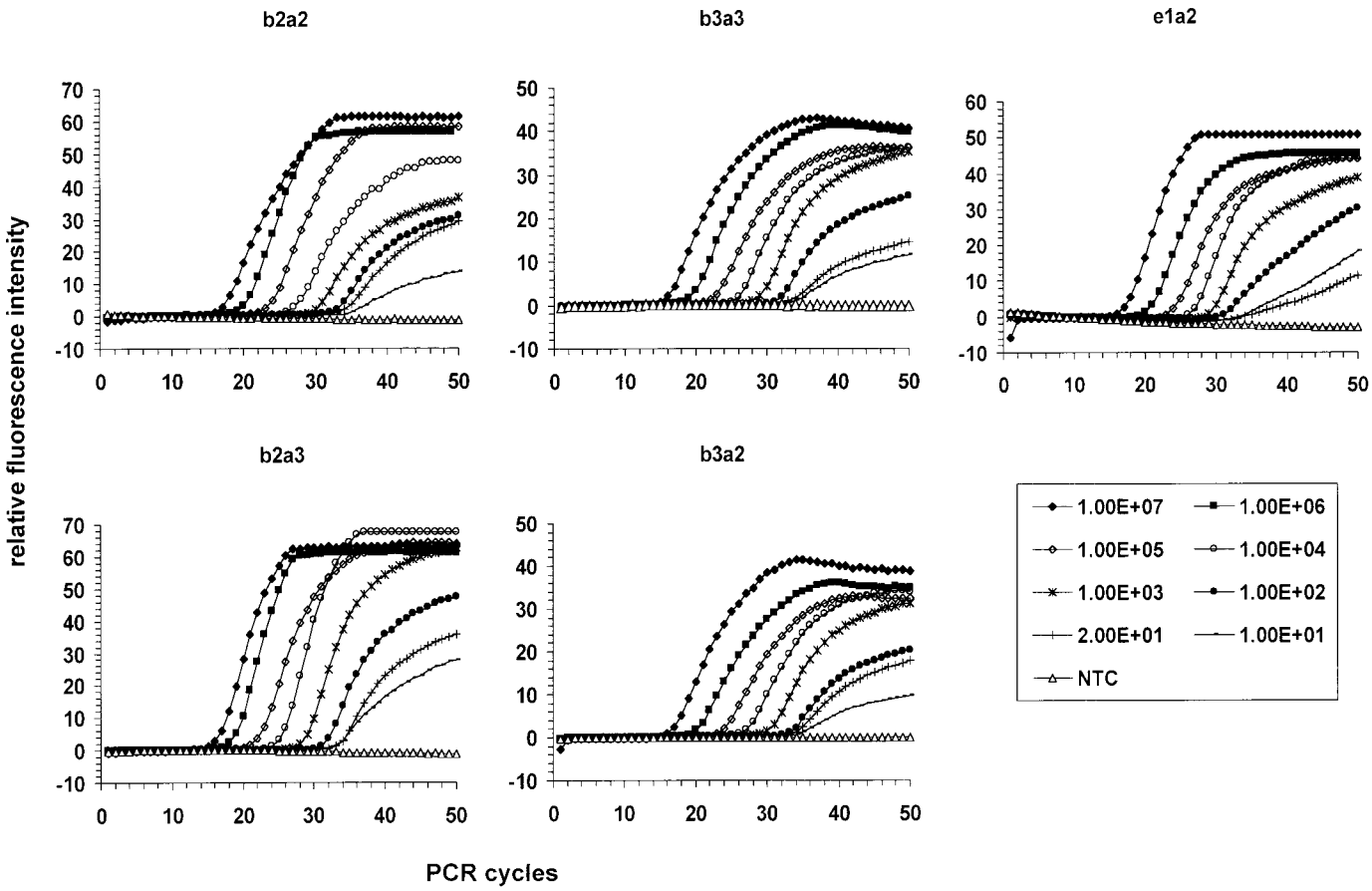

Fig. 2. Fluorescence data of all five bcr/abl fusion standards generated by the LightCycler instrument. The relative change in fluorescence during cycling is given on the *Y axis*. *Inset*, absolute numbers of starting templates (*1.00E+07*, 10^7; *1.00E+05*, 10^5; and so on). *NTC*, nontemplate controls.

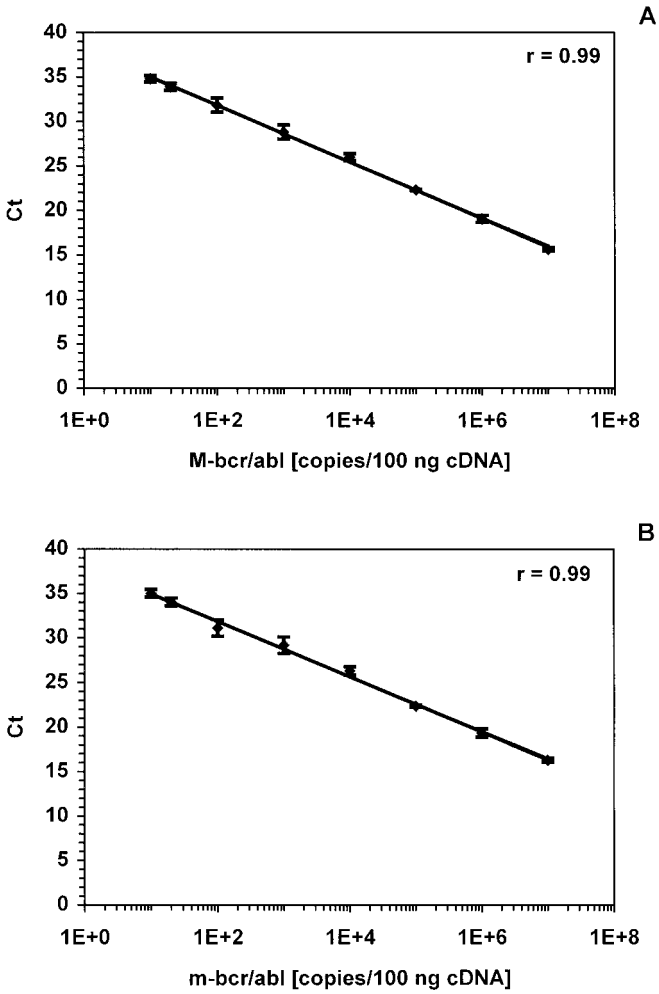

Fig. 3. Calibration curves of M-bcr/abl and m-bcr/abl fusions. *A*, mean threshold cycle (*Ct*) for repetitive analysis of four M-bcr/abl fusion standards (b2a2, b2a3, b3a2, and b3a3); $r = 0.99$. *B*, calibration curve for m-bcr/abl (e1a2); $r = 0.99$.

the correlation coefficient r was >0.99, indicating a precise log-linear relation in the range between 10 and 10^7 copies per 100 ng of cDNA.

Because the amplicons of the four M-bcr/abl fusions exhibit different base sequences and sizes (range, 127–373 bp), PCR efficiency may vary from fusion to fusion. However, considering all dilution steps of all M-bcr/abl calibrators, the mean SD of the *Ct* value for each separate dilution was 0.43 cycles. This deviation was not significantly different from the SD within one breakpoint (SD = 0.41 cycles), indicating a comparable reaction efficiency. Thus, all four M-bcr/abl fusions can be measured with one primer pair and one fluorogenic probe in one reaction. Given a theoretical reaction efficiency of 0.9 (*i.e.*, 90%), ~3.59 cycles are necessary to reach a 10-fold amplification of the initial molecule number, according to the equation: $c = (\ln n_c - \ln n_i)/\ln (1 + e)$, where c is the number of cycles, e is the reaction efficiency, n_i is the initial molecule number, and n_c is the ending number of molecules. Our PCR results are fairly consistent with this assumption, which emphasizes the accuracy of the experimental system: over the entire range from 10^7 to 10 copies per 100 ng of cDNA of all five fusions, the mean *Ct* interval between the 10-fold dilution steps was 3.20 ± 0.31 cycles.

To compare absolute amounts of template molecules obtained by both real-time fluorescence detection systems, bcr/abl copy numbers of 10 preselected bcr/abl-positive samples were correlated. As can be seen from Fig. 4, values in the range from 1×10^5 to 7×10^3 copies per 100 ng of cDNA generated by both systems correlated fairly well ($r = 0.71$). Furthermore, none of 10 samples tested negative by nested PCR were positive in the LightCycler PCR.

DISCUSSION

The clinical usefulness of molecular quantitation of bcr/abl fusion transcripts has long been investigated, and it appears that quantitative fluorescence PCR is one of the most promising methods for monitoring the course of disease in CML patients. This recently introduced technique allows rapid analysis of absolute template amounts without any post-PCR steps, making it very feasible for routine clinical diagnosis. We and others have shown that individual bcr/abl transcript kinetics can be sensitively detected by this approach and that quantitative results are highly indicative of progressive or remissive CML (15).[3] The now available LightCycler apparatus may provide additional methodological advantages, namely, a further increase in PCR speed by using a newly developed heating/cooling technique. However, the HybProbe format, which was developed to be used in the LightCycler, may be inconvenient. For each of the detection probes to be used with maximum specificity, each of the HybProbes should cover at least 25 nt of the target sequence. Furthermore, hybridization efficiency of both probes should be comparable to ensure simultaneous annealing of the two oligomers. Therefore, one is restricted to target sequences, which allow HybProbe design within at least 50 nt. Probe design may, therefore, be complicated by divergent G/C content of HybProbes, leading to different hybridization characteristics. If hybridization efficiency differs significantly between the two fluorescent probes, this may result in a higher background signal and, consequently, in a loss of sensitivity. Because secondary structures of the primers and target probes may have an substantial effect on hybridization and, thus, on PCR specificity and sensitivity, fluorescent PCR must be optimized such that annealing of probes and annealing of primers are equally efficient. Thus, it is recommended that primers with similar melting temperatures be used and that, to ensure maximum probe hybridization during the annealing/extension step, the melting temperature of the probe be at least 5°C higher than those of the primers. Depending on the target sequence, these criteria cannot always be completely satisfied and may be even more difficult to fulfill when four oligonucleotides must be considered in the reaction, such as in the HybProbe format. Finally, the more oligonucleotides that are in the reaction mix, the higher the probability of unspecific

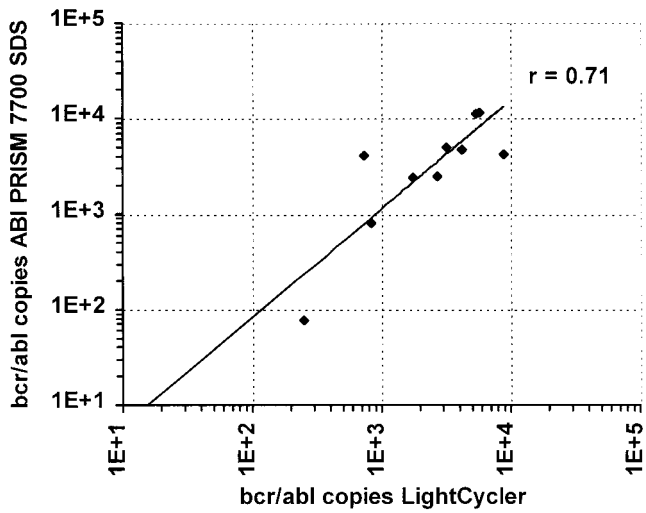

Fig. 4. Correlation between quantitative bcr/abl results obtained by the LightCycler (*X axis*) and the ABI PRISM 7700 SDS (*Y axis*); $r = 0.71$.

bindings, including polymerization of primers and probes. In contrast, TaqMan probes are easier to design and require only one additional oligonucleotide to be considered in PCR. In our system, TaqMan fluorescent dyes were positioned within distances of 9 and 19 nt. However, as we described elsewhere, the quenching effect is not altered when fluorescent dyes are positioned within a distance of <30 nt (16).

Both systems have different advantages that are independent from the probe design. The LightCycler, by using a newly developed heating and cooling technique, produces quantitative results extremely rapidly (17). Furthermore, this system allows determination of melting curves using double-stranded specific fluorophores. On the other hand, data analysis is more convenient with the ABI PRISM 7700 SDS because a constant algorithm is used to calculate initial template number. In contrast, in the LightCycler, no passive reference is measured, and therefore, no background signal can be subtracted. Fluorescence data must be analyzed by individualized procedures. Thus, the high degree of analytical automation in the ABI PRISM 7700 SDS may be advantageous for standardized routine diagnostics, whereas LightCycler technology offers a more rapid and flexible procedure. However, these differences are mainly due to different computer software and, thus, may change quickly due to the invention of new analytical software versions.

We conclude that, with our protocol, TaqMan chemistry can be used for quantitative bcr/abl RT-PCR in the ABI PRISM 7700 SDS as well as in the LightCycler. Both assays exhibit comparable characteristics and allow sensitive quantitation of bcr/abl fusion transcripts in clinical samples. Future studies should aim to optimize and to standardize pre-PCR sample preparation to further increase the degree of reliability in real-time fluorescence-based RT-PCR techniques for the quantitation of bcr/abl fusion RNA.

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