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Rapid quantitative detection of BCR-ABL transcripts in chronic myeloid leukemia patients by real-time reverse transcriptase polymerase-chain reaction using fluorescently labeled probes

PASCUAL BOLUFER,* GUILLERMO F. SANZ,° EVA BARRAGÁN,* MIGUEL A. SANZ,° JOSE CERVERA,° ENRIQUE LERMA,* LEONOR SENENT,° ISABEL MORENO,* MARÍA D. PLANELLES#

**Laboratory of Molecular Biology (Department of Clinical Pathology), °Department of Hematology, Hospital Universitario La Fe, and #Laboratory of Histocompatibility, Centro de Transfusión Comunidad Valenciana, Valencia, Spain*

ABSTRACT

Background and Objectives. **The limited value of qualitative reverse transcription polymerase chain-reaction (RT-PCR) for monitoring chronic myeloid leukemia (CML) patients has prompted the development of quantitative assays. We have developed a quantitative real-time PCR (QC-PCR) method in the LightCycler, based on the use of fluorescently labeled probes (HybProbes), to estimate BCR-ABL fusion gene transcripts in samples from CML patients.**

Design and Methods. **Fifty-two samples (45 peripheral blood, five bone marrow, and two apheresis product samples) from nine patients with CML were analyzed. Seven patients were studied at diagnosis and during follow-up after hematopoietic stem cell transplantation (HSCT), whereas two were evaluated only after HSCT. The PCR reaction was carried out in capillary tubes in a final volume of 10μL, using 2 μL cDNA, the Mensik *et al.* primers, and two HybProbes. The results for BCR-ABL were normalized with reference to ABL. The PCR program is completed in only 45 min.**

Results. **The sensitivity attained allowed the detection of rearrangements at dilutions of between $5\text{-}10^{-4}$ and 10^{-5} K562 cDNA. The within-assay coefficient of variation was 11% for BCR-ABL, and 9% for ABL. A greater than 2 log reduction in the BCR-ABL/ABL ratio was evident shortly after transplantation in all allografted patients.**

Interpretation and Conclusions. **We may conclude that the TaqMan probe technology can be easily adapted to HybProbes with equivalent results. Besides, the results of BCR-ABL quantification in the follow-up of patients clearly confirm that real-time PCR with HybProbes is a reliable and sensitive method for monitoring minimal residual leukemia after HSCT in CML patients.**

Key words: quantitative PCR, BCR-ABL, chronic myeloid leukemia, minimal residual disease, hematopoietic stem cell transplantation

Correspondence: Pascual Bolufer Gilabert, M.D., Laboratorio de Biología Molecular, (Lab Hormonas), C Maternal, Hospital Universitario La Fe, Avda Campanar 21, 46009 Valencia, Spain. Phone: international +34-96-3987377 – Fax: international+34-96-3868730 – E-mail: bolufer_pas@gva.es

The hallmark of chronic myeloid leukemia (CML) is the BCR-ABL fusion gene, which is usually formed as a result of the t(9;22) translocation. CML serves as a paradigm for the utility of molecular methods to diagnose malignancy, or to monitor patient response to therapy.[1] Reverse transcription polymerase chain reaction (RT-PCR) for BCR-ABL mRNA is by far the most sensitive and, consequently, the most adequate method with which to monitor minimal residual disease (MRD). However, the limited value of qualitative PCR for monitoring CML patients after therapy prompted the development of competitive BCR-ABL assays, to quantify the BCR-ABL transcripts after hematopoietic stem cell transplantation (HSCT)[2-4] or treatment with interferon (α-IFN).[5,6]

With the recent advent of real-time PCR technology, several quantitative real-time RT-PCR methods for BCR-ABL quantification have been published. Some of them are based on the use of the TaqMan™ probe with ABI/Prism™ 7700 equipment (Perkin Elmer);[7,8] others have been developed for the recently introduced LightCycler apparatus (Roche), using TaqMan™ technology[9] or hybridization probe (HybProbe) chemistry.[10]

HybProbe chemistry consists in two adjacent probes in a head-to-tail orientation, spaced by one to four nucleotides. The probes hybridize to adjacent sequences. One of the probes is labeled at its 3' end by a donor dye (generally fluorescein). The other probe is labeled with an acceptor molecule at its 5' end (generally LC Red640 or 705), and is phosphate-blocked at the 3' end. When both probes are hybridized to their target sequences, the emitted light of the donor is transmitted to the acceptor fluorophore by Förster resonance energy transfer (FRET),[11] and the Red640 emitted fluorescence (640 nm) can be detected. The intensity of the emitted fluorescence increases in parallel with the target DNA formed in the PCR. The LightCycler probes offer the advantage

over the TaqMan™ probe of not requiring hydrolysis and, therefore, no additional extension of the PCR times (annealing-elongation ≤ 12 s). It is, therefore, possible to take advantage of the high-speed thermal cycling of the LightCycler, and complete the PCR program in only 45 min. The present study reports a new method for BCR-ABL quantification adapted to the LightCycler equipment. The method was designed using the original primers of Mensik *et al.*[7] for BCR-ABL quantification in the ABI/Prism™ 7700, but substituting the TaqMan™ probes with HybProbes methodology.

The results obtained support the hypothesis that TaqMan™ probe technology can be easily adapted to the HybProbes, which confers the advantage of developing faster PCR assays, especially when they are performed in very fast cycling equipment. We also show here, in a retrospective analysis of nine patients after HSCT, that normalized BCR-ABL/ABL expression can be definitely quantified over time using HybProbe chemistry and QC-PCR performed in the LightCycler.

Design and Methods

Patients

Overall, 52 samples (45 peripheral blood [PB], five bone marrow [BM], and two apheresis product samples) from nine patients (median number of samples per patient was six; range 4–9) with CML (six b3a2, and three b2a2), were evaluated by QC-PCR. Seven patients were studied at diagnosis, or shortly thereafter, and during follow-up after HSCT. Two were evaluated only after HSCT. The median follow-up was eight months (range 4–18 months). All samples belonging to a particular patient were processed in the same assay. There were five women and four men, and their median age at transplant was 40 years (range 18–52 years). Five patients had received an HLA-identical allogeneic BM transplant (BMT) and two had received an HLA-mismatched unrelated donor umbilical cord blood transplant (UD-UCBT). One patient had received an autologous PB stem cell transplant (APBSCT), with stem cells collected after chemotherapy, and was receiving IFN post-transplant in an attempt to avoid or delay relapse. The remaining patient had undergone an HLA-identical sibling BMT seven years before starting this study. He first experienced a cytogenetic relapse, and afterwards a hematologic relapse, and was treated with IFN.

Reagents

Taq Start™ antibody (PT 1576-1) (Clontech, Palo Alto, CA, USA, Catalog #5400-1); Light Cycler™-DNA Master Hybridization Probes (Roche Molecular Biochemicals, Indianapolis, IN, USA, Cat. No 2015102); FastStart Light Cycler™-DNA Master Hybridization Probes (Roche Molecular Biochemicals, Indianapolis, IN, USA, Cat. No. 3003248); Uracil-DNA-Glycosylase, heat-labile IU/µL (Roche Molecular Biochemicals Mannheim, Germany, Cat. No 1775367); Light Cycler™-Capillaries 8x96 (Roche Molecular Biochemicals, Mannheim, Germany, Cat. No. 190939); TOPO™ TA Cloning® (Invitrogen BV, 970 VT Groningen, The Netherlands, K4600-01).

Cloning of PCR products

Quantification involved the use of standard curves that had been prepared with plasmids containing specific sequences of the gene or rearrangements studied. We cloned the PCR products obtained by amplifying patient samples (b3a2, b2a2 or ABL) into the pCR® II-TOPO vector, using the protocol provided with the TOPO™ Cloning® kit (Invitrogen BV). Following this procedure, we prepared the plasmids pCR II-TOPO (b3a2BCR-ABL) and pCR II-TOPO (b2a2BCR-ABL), which contain the inserts b3a2 and b2a2, respectively, and the plasmid pCR II-TOPO (ABL), which contains ABL.

Nucleic acid isolation (RNA and DNA)

Mononuclear cells were isolated from ethylenediaminetetra-acetic acid (EDTA)-anticoagulated peripheral blood by Lymphoprep (Nycomet, Pharma AS) density gradient centrifugation. The cells collected (mean number 5×10^6) were resuspended in guanidinium thiocyanate solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7, containing 5 g/L sarcosyl (N-lauroylsarcosine), and 0.1 M 2-mercaptoethanol) and stored at –80°C until RNA extraction. RNA was extracted following the guanidinium-thiocyanate, phenol-chloroform procedure of Chomczynski and Sacchi.[12]

To study the chimerism status of patients, DNA was extracted using a salting out procedure[13] from donor and recipient PB or BM cells before allogeneic HSCT, and from patient BM or PB after transplant.

cDNA synthesis

One to three micrograms of RNA were transcribed to cDNA in 25 µL reaction volumes, with MoMuLV-reverse transcriptase (Promega, Madison, WI, USA), using random hexamer primers. The RNA was first incubated at 70°C for 5 min, and then the reagents added. The reaction was performed for 10 min at 22°C, then at 42°C for 60 min and finally at 95°C for 5 min to inactivate the reverse transcriptase.

Table 1. Primers and labeled probes.

Primers/probes	*Sequences*	*Mapping(Version) Positions*
Primers		
BCR-ABL		
Bcr-abl For	5' CgggAgCAgCAgAAgAAgTgT-3'	BCR(X02596),3072-3089
Bcr-abl Rev*	5'-CgAAAAggTTggggTCATTTTC-3'	ABL(X16416),346-325
ABL		
A2+	5'-TTCAgCggCCAgTAgCATCTgACTT-3'	ABL(X16416),233-257
CA3-*	5'-CTgTTgACTggCgTgATgTAgTTgCTT-3'	ABL(X16416), 509-483
Hybridization probes		
BCR-ABL		
Bcr-abl 3FL	5'- CggCCAgTAgCATCTgACTTTgAgC-F	ABL(X16416),238-262
Bcr-abl 5LC	5'-LC Red640-TCAgggTCTgAgTgAAgCCgCTC-P	ABL(X16416),264-286
ABL		
bcr-abl 3FL	5'-CCAAggAAAACCTTCTCgCTggACCC-F	ABL(X16416),296-321
bcr-abl 5LC	5'-LC Red640-TgAAAATgACCCCAACCTTTTCgTTgCAC-P	ABL(X16416),324-352

Abbreviations: P=phospate, F= fluorescein.

Standard curve and controls

For the construction of standard curves for BCR-ABL and ABL, plasmids pCR II-TOPO (b3a2*bcrabl*) (28 ng/µL) and pCR II-TOPOABL (84.5 ng/µL) were prepared as 10-fold serial dilutions in water, from 10^{-3} to 10^{-7}. These were stored at –20°C until use.

Sensitivity and reproducibility studies were carried out using dilutions of the cell line K562, or dilutions of the RNA extracted from cells.

Real time PCR

For BCR-ABL we used the *Bcr-abl forward* primer and a modified *Bcr-abl reverse* (Bcr-abl rev*) primer of Mensik *et al*.[7] The fluorogenic 3' hybridization probe (*Bcr-abl 3FL*), labeled with fluorescein, and the 5' probe (*Bcr-abl 5LC*), labeled with LC Red640, compatible with the Mensik primers, were designed and synthesized by TIB MOLBIOL (Berlin, Germany) (Table 1).

For ABL, the A2 and a modified CA3-* primers of Cross *et al.*[14] were used. The probes *bcrabl* 3FL, fluorescein-labeled at the 3' end, and *bcr-abl 5LC*, labeled with LC Red640 at the 5' end and phosphate-blocked at its 3' end, were also designed and synthesized by TIB MOLBIOL (Table 1). The PCR was performed in 10 µL final volume, using 1 µL of the LightCycler DNA Master Hybridization Probes mastermix. More recently, we have used the newly introduced *hot-start* system, FastStart Light Cycler™-DNA Master Hybridization Probes (Roche Diagnostics). This mixture contains buffer, dNTPs in which dTTP is replaced by dUTP, 1 mM $MgCl_2$, and active Taq DNA polymerase, in the case of LightCycler DNA Master Hybridization Probes, or modified inactive Taq in FastStart. In those assays performed with LightCycler DNA Master Hybridization Probes, 1 µL of the mixture was preincubated with 0.16 µL of the anti-Taq antibody (TaqStart, Clontech), for 5 min at room temperature. The BCR-ABL primers (*Bcr-abl* for and *Bcr-abl rev**) were used at 0.45 µM concentrations, and those for ABL (A2 and CA3-*) at 0.52 µM. All the fluorescent probes, both, for BCR-ABL (*Bcr-abl* 3FL and Bcr-abl, 5LC) or for ABL (*bcrabl* 3FL and *bcrabl* 5LC), were used at 0.2 mM final concentration.

We used $MgCl_2$ at 3 µM final concentration, and added 0.5 U of heat labile uracil-DNA glycosylase (UDG) to eliminate PCR contamination from previous reactions. Two microliters of the points of standard curve or cDNA samples were used in the BCR-ABL assay, and 1 µL for the ABL assay. Amplifications were performed in the LightCycler in a three-step procedure. The first step consisted of incubation at 32°C for 5 min, to allow the UDG to degrade any possible contamination from a previous PCR. This was followed by an incubation at 94°C for 10 min to activate Taq (when the FastStart system was used), or for 1 min (when LightCycler DNA Master was used).

The amplification was performed for 45 cycles, each one involving annealing at 60°C for 9 s, elongation at 72°C for 9 s, and denaturation at 94°C for 0 s. Fluorescence was measured at the end of the annealing step at the F2 (640 nm)/F1 (530 nm) channel ratio. The calculations were also carried out with the software (LightCycler 3) provided with the LightCycler apparatus. The software included with the equipment, based on the established relationship between the cycle threshold (C_T) and the logarithm of the initial number of target copies (N) present in the sample[15,16] fits an empirical straight-line with the points of the standard curve. This allows estimation of N for each sam-

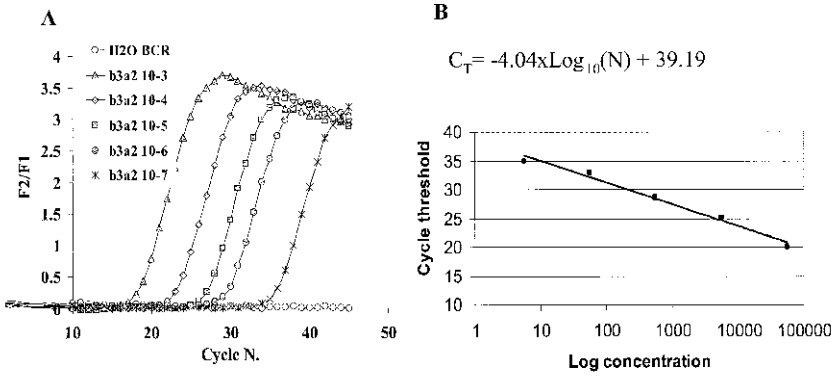

Figure 1. (A) BCR-ABL standard curve obtained by 10-fold dilutions of the plasmid pCR II-TOPO (b3a2BCR-ABL). (B) Straight line obtained from cycle threshold (C_T) and concentration of each point of the standard curve.

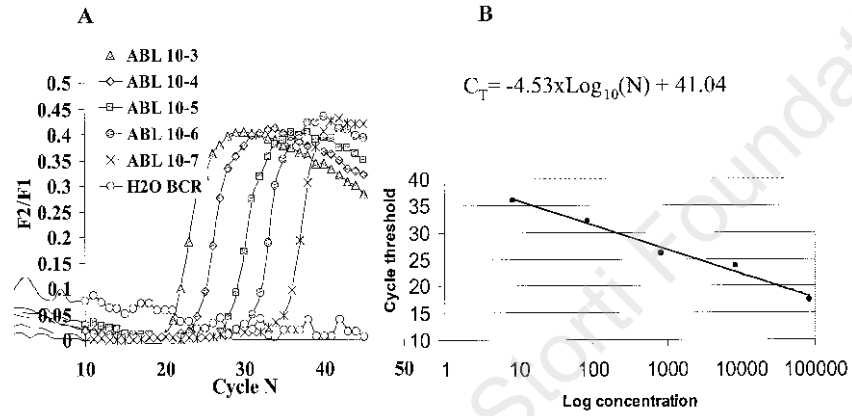

Figure 2. (A) ABL standard curve obtained by 10-fold dilutions of the plasmid pCR II-TOPO (ABL). (B) Straight line obtained from cycle threshold (C_T) and concentration of each point of the standard curve.

ple on the basis of its C_T, for both BCR-ABL and the control gene ABL. The ABL transcript assessed with the PCR reaction represents the sum of the amounts of the ABL transcript plus the BCR-ABL transcripts.

The initial concentration of the target molecules in each sample (N) was expressed in terms of femtograms of plasmid BCR-ABL, or ABL per microliter of cDNA. Normalized levels were calculated as the ratio BCR-ABL/ABL, and expressed as a percentage.

Other studies

The chimerism status after allogeneic HSCT was evaluated by three different techniques: PCR with variable number tandem repeats (PCR-VNTR), PCR with sequence-specific probes (PCR-SSP) and PCR with single specific oligonucleotide probes (PCR-SSO), in those cases of HLA antigen mismatches, and fluorescence *in situ* hybridization (FISH), in those cases with donor-recipient sex disparity. The presence of BCR/ABL rearrangement was also evaluated by FISH methodology.

Statistics

The Mann-Whitney U test was used for comparing ranks, and Pearson's correlation coefficient for studying possible correlations between different variables. All descriptive statistics and tests were calculated using the statistical package SPSS 8.0. Values of p below 0.05 were considered statistically significant. To fit the straight lines of the standard curves, the software provided with the equipment was used (LightCycler 3).

Results

Standard curves

The regression coefficients achieved in five ABL and BCR-ABL consecutive standard curves were all greater than 0.96. For BCR-ABL, we estimated a mean±SD slope of –4.04±0.40, with mean±SD intercept of 39.19±2.01 (Figure 1). For ABL, a slope of –4.53±0.49 and intercept of 41.04±2.19 (Figure 2) were calculated.

The means of C_T for each point in the five consecutive standards for BCR-ABL and ABL were almost steady (SD < 0.7 cycles).

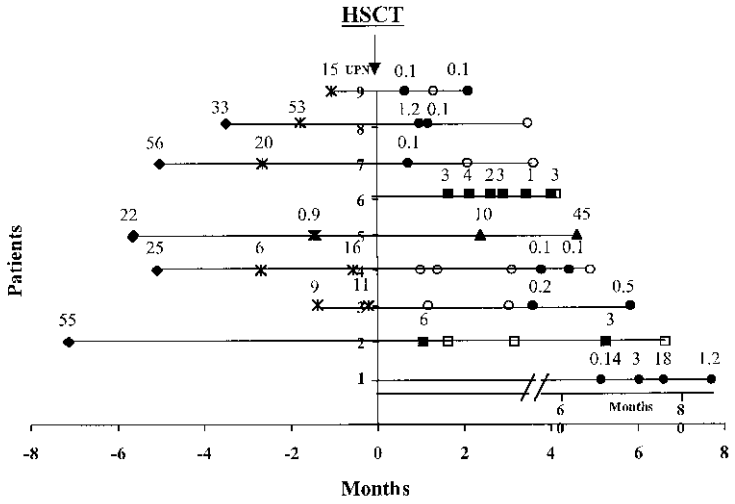

Figure 3. Quantitative monitoring of BCR-ABL transcripts of the CML patients studied. Open dots and full dots indicate negative and positive, respectively. The number over the full dots indicates the PCR quantitative analysis expressed as BCR-ABL / ABL in percentage.
HSCT = hematopoietic stem cell transplant. UPN = unique patient number. Diamond = diagnosis; round circle = allogeneic HSCT; square = unrelated donor umbilical cord blood transplantation; triangle = autologous peripheral blood stem cell transplantation; stars = samples before HSCT.

Quality of amplified products

Some amplification products produced by the LightCycler were checked by electrophoresis on a 2% agarose minigel, verifying both the absence of artifacts and that the size of the amplified products corresponded to the expected sizes (310 bp for b3a2, 239 bp for b2a2, and 250 bp for ABL).

Sensitivity

The method has unequal sensitivity for the transcripts b3a2 and b2a2. Hence, it detects less than 5.6 fg plasmid pCR II-TOPO (b3a2BCR-ABL), equivalent to 1,200 copies, and detects less than 0.186 fg pCR II-TOPO (b3a2BCR-ABL), equivalent to 40 plasmid copies.

When the sensitivity was studied in serial 10-fold dilutions (from 10^{-2} to 10^{-5}) of the cell line K562, diluted in white blood cells, the method was capable of detecting one single K562 cell among 50,000 normal white cells. Ten-fold aqueous dilutions of cDNA obtained by RT of RNA from K562 cells in the same range (from 10^{-2} to 10^{-5}) were also used to test the sensitivity of the method. A dilution of $5x10^{-4}$ was detectable when 1 µg RNA was transcribed, and this increased to at least 10^{-5} when 3 µg RNA were transcribed.

Reliability of the assay

The intra-assay reproducibility was studied by repeating the analysis of the same sample 10 times in the same assay. For BCR-ABL C_T, a mean±standard deviation (SD) of 29.97±0.19 cycles was estimated, which reflects a coefficient of variance (cv) of 0.64%. This result corresponds to 70.89±7.93 fg/µL cDNA, which represents a within assay cv of 11%. For ABL C_T, we obtained a mean±SD of 26.76±0.13 cycles, with a cv of 0.49%, or 1,476±133 fg/µL cDNA, which reflects a cv of 9%.

Quantitative BCR-ABL and patient profiles

BCR-ABL/ABL was analyzed before HSCT in 11 samples (nine PB, and two BM) from six allografted patients. Five samples were obtained at diagnosis (four PB, and one BM), and six samples (five PB, and one BM) from six patients who were under treatment with hydroxyurea. BCR-ABL/ABL showed a median ratio of 20% (range 6-56%). A clear correlation between BCR-ABL/ABL ratio and absolute leukocyte count was not evident. The BCR-ABL/ABL ratio significantly decreased after allogeneic HSCT in all six allografted patients with samples available before and after transplant. The BCR-ABL/ABL median ratio was 0.1% (range 0/-6.2%) in the 31 samples, which represented a greater than 2 log reduction in the BCR-ABL/ABL ratio at two months after transplantation (p<0.001). This was mainly due to a strong reduction in BCR-ABL transcripts, that changed from 554 (range 275-5,695) fg BCR-ABL/µL cDNA before allogeneic HSCT, to 1.1 (range 0–161) after allogeneic HSCT.

A small, but significant, decrease after allogeneic HSCT (p=0.024) in ABL transcripts was also observed, which could be justified by the significant correlation observed between BCR-ABL and ABL transcripts (Pearson's correlation = 0.67; n = 42; p<0.001). The median BCR-ABL/ABL percentage after allogeneic HSCT in these seven patients was 0.10 (range 0-6). In all of the patients, one or more samples (n=14) tested BCR-ABL/ABL negative shortly after transplant, by qualitative and QC-PCR (Figure 3). However, in three cases, after one or more

negative samples there was reappearance of BCR-ABL transcripts followed by a sudden return to a PCR negative status. In two of them, BCR-ABL negativity was only evident after development of graft-versus-host disease (GVHD). Full donor chimerism, by any of the three methods employed during the study, and the absence of BCR-ABL rearrangement by FISH, was always observed when the BCR-ABL/ABL ratio by QC-PCR dropped below 1%. However, when the BCR-ABL/ABL ratio by QC-PCR was between 1% and 7% some cases showed full donor chimerism by FISH, PCR-VNTR and/or PCR-SSP/PCR-SSO whereas a mixed chimerism was demonstrated in others. Low numbers and the vastly different sensitivities of the three techniques used to monitor chimerism status after allogeneic HSCT precluded meaningful comparisons between the results of quantitative RT-PCR and these methods.

Discussion

The first reports of BCR-ABL quantification were developed in the ABI 7700[7,8] by TaqMan™ technology. This technology exploits Taq 5' exonuclease activity, which hydrolyzes the hybridized TaqMan™ probe during the elongation step. This hydrolysis releases the labeled reporter dye, generally 5' TET (tetrachloro-6-carboxyfluorescein), from the quencher dye, generally 3' TAMRA (6-carboxytetramethylrhodamine), conjugated to the probe. Henceforth, the extension of the PCR products can be followed by monitoring the fluorescence intensity of the light emitted by the laser-excited, released reporter molecule.[17] However, to achieve the 5' nuclease activation, elongation-annealing times of at least one minute are generally required.

In spite of the short time that has elapsed since the introduction of the LightCycler, some reports have already demonstrated its compatibility with TaqMan™ technology.[9] However, the use of these probes has the drawback of requiring longer incubation times with regard to the set of hybridization probes. The present study takes advantage of the high speed of the LightCycler (<40 s/cycle), that results from the fast ramp rate during heating and cooling. This, combined with the HybProbe technology, allows completion of the assay in only 45 minutes. Moreover, the fast ramp times, and the brevity of the annealing and elongation steps, improve the specificity, yielding very clean PCR products. Nonetheless, the characterization of transcripts *b3a3* or *b2a2* requires electrophoresis of the PCR products. However, if PCR is performed using DNA SYBR Green I, instead of the specific HybProbes, the transcripts *b3a2* or *b2a2* could be characterized by their melting peaks. A previous report has already validated the utility of HybProbes in real time PCR for BCR-ABL quantification in the LightCycler.[10] The present study shows that the methods designed for TaqMan technology can be easily converted to HybProbe technology with equivalent results. The standard curves were very reproducible in successive assays, yielding very consistent C_T for each one of the points on the standard curves, for both BCR-ABL and the control gene ABL. The standard straight lines that were fitted had very high correlation coefficients, and similar slopes and intercepts.

The sensitivity of the method (one single K562 cell among 50,000 normal white blood cells, or at dilutions between 4×10^{-4} and at least 10^{-5} of cDNA) is similar to that reported by Mensik *et al.*[7] using TaqMan probes (one malignant cell in 10^5 normal cells), but somewhat lower than in other studies.[4,10] However, the method has the advantage of accomplishing the entire assay in only 45 min. Moreover, we observed a similar sensitivity using SYBER Green I (data not shown). The intra-assay precision obtained here is very similar to that reported in previous studies of real-time PCR.[7,10]

This precision is adequate to monitor the samples of each patient reliably. As expected, and in accordance with previously published studies,[8,18] there was a significant decrease in the absolute number of BCR-ABL transcripts, and in the BCR-ABL/ABL ratio after HSCT. Although analyzed in a small number of patients, our results suggest that the pattern of disappearance of BCR-ABL transcripts is not uniform. In some instances, after one or more negative samples there was a reappearance of BCR-ABL transcripts followed by a sudden return to a PCR negative status as has been shown in other series.[18] This phenomenon was temporally related to the emergence of graft-versus-host disease in some cases.

Analysis of the method established here allows us to conclude that the TaqMan probe technology can be easily adapted to HybProbes with equivalent results. Besides, our preliminary results of BCR-ABL quantification in a limited series of allografted patients confirm that real-time PCR with hybridization probes is a reliable and sensitive method for monitoring minimal residual leukemia after HSCT in CML patients. However, before real-time PCR is used in clinical situations for anticipating and offering therapy before cytogenetic relapse, evaluation of this methodology in a large, prospective series of patients is clearly required.

Contributions and Acknowledgments

PB was the principal investigator: he designed the study and was partly responsible for the funding, super-

vision and critical revision of the manuscript. GFS and MDP were responsible for the drafting of the manuscript and clinical management of patients. MAS was responsible for part of the funding of the project and critically revised the manuscript and gave final appoval for its submission. EBG and IMM were responsible for setting up quantitative procedures and drafted the manuscript. JC and LS were responsible for the FISH and chimerism studies and co-operated in the drafting of the manuscript. EL was responsible for the quantitative assessments and co-operated in the drafting of the manuscript. The order of the authorship was made according to the contribution given to the study.

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Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping withg previous papers.

Potential implications for clinical practice

- The results show that methods based on TaqMan technology can be easily adapted to the HybProbe. Moreover, HybProbe methods seem equally reliable but to be have the advantage of taking less time.
- The development of reliable quantitative PCR might contribute to a better understanding of the kinetics of the leukemic clone in CML and, therefore, to a more effective understanding of the potential benefits of the different therapeutic regimes applied to control and cure the disease.

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