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Manifold-assisted Reverse Transcription-PCR with Real-Time Detection for Measurement of the *BCR-ABL* Fusion Transcript in Chronic Myeloid Leukemia Patients

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Background: ***BCR-ABL* fusion mRNA expression in bone marrow or peripheral blood can be used as a measure of minimal residual disease in patients with chronic myeloid leukemia (CML).**

Methods: **We used an oligo(dT)-coated manifold support to capture the mRNA directly from the cell lysate. After reverse transcription, the cDNA was eluted from the manifold support, and *BCR-ABL* and *GAPDH* mRNAs were quantified in real time using the TaqMan fluorogenic detection system.**

Results: **The detection limit of the method was one positive K562 cell among 10^5 negative cells. *GAPDH* was chosen as a reference gene based on the low variation between samples from different stages of the disease and the low signal in the absence of reverse transcription. The day-to-day variation of the method (CV) was 32%. In 43 blood samples from 13 CML patients, mRNA quantification agreed well with cytogenetic data.**

Conclusions: **The proposed procedure constitutes a reproducible and sensitive *BCR-ABL* mRNA quantification method and is suitable to monitor minimal residual disease in CML patients.**

Chimeric genes resulting from chromosomal translocations can be used as disease-specific markers for the malignant clone in several hematological malignancies *(1)*. Up to 90% of the patients with chronic myeloid leukemia (CML)[3] carry the cytogenetic abnormality known as the Philadelphia chromosome, which arises from a translocation between chromosomes 9 and 22 *(2)*. As a result, the 5′ end of the *BCR* gene on chromosome 9 becomes fused to the 3′ end of the *ABL* gene on chromosome 22 *(3)*. Cells carrying the translocation can be identified by cytogenetic analysis, fluorescence in situ hybridization, Southern blotting, or by reverse transcription-PCR (RT-PCR) of the fusion transcript [reviewed in Ref. *(4)*]. Two predominant hybrid transcripts are observed in CML, joining either exon b2 or b3 of the *BCR* gene to exon 2 of the *ABL* gene. Quantitative competitive RT-PCR has been valuable for monitoring patients with CML after bone marrow transplantation *(5–8)*. Consistently low or decreasing *BCR-ABL* mRNA expression constitutes a good prognostic factor. However, current RT-PCR-based technologies are difficult to implement in routine laboratories.

The advent of methods to monitor DNA amplification reactions in real time, such as the 5′-nuclease assay *(9)*, has made mRNA quantification by RT-PCR simpler and more accurate. The 5′-nuclease assay takes advantage of the 5′-nuclease activity of *Taq* DNA polymerase to cleave a dual-labeled probe hybridizing to the amplified fragment during the extension phase *(10)*. The cleavage reaction separates the two fluorophores, abolishing fluorescence resonance energy transfer, and producing increased fluorescence. The increase in fluorescence is proportional to the target accumulation and can be measured in real time *(11)*. The fractional amplification cycle at

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[3] Nonstandard abbreviations: CML, chronic myeloid leukemia; RT-PCR, reverse transcription-PCR; C_T, threshold cycle; ASCT, autologous stem cell transplantation; BMT, bone marrow transplantation; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; α-IFN, α-interferon; and HU, hydroxyurea.

which fluorescence exceeds baseline fluorescence is called the threshold cycle (C_T). The C_T value is recorded as a measure of the number of target molecules in the amplification reaction.

RNA typically is isolated through extraction with acid guanidinium-phenol-chloroform *(12)*, a method that is relatively labor-intensive and requires the use of hazardous chemicals. Simultaneous processing of numerous samples is difficult and time-consuming, limiting the applicability of RT-PCR in routine laboratories. We recently developed an oligo(dT)-coated manifold support that allows mRNA to be isolated directly from cell lysates by hybridization followed by transfer through the different washing and enzymatic steps of the assay with minimal pipetting *(13)*.

In the present report, we describe a streamlined and rapid method to quantify *BCR-ABL* transcripts, where the mRNA in cell lysates is captured onto a manifold solid support and quantified in real time using the TaqMan fluorogenic detection system. The whole procedure can be completed in a few hours. We used the method to follow *BCR-ABL* transcript expression in peripheral blood from 13 CML patients, comparing the results to cytogenetic data from bone marrow smears.

Materials and Methods

PATIENTS AND SAMPLES

We analyzed peripheral blood mononuclear cells from 13 patients with CML (Table 1). Patients 1 and 2 received autologous stem cell transplantation (ASCT) during chronic phase, and the remaining patients received allogeneic bone marrow transplantation (BMT) during chronic phase.

After informed consent, peripheral blood was collected from CML patients in EDTA tubes, and mononuclear cells were separated by Ficoll density gradient centrifugation. The cells were washed in phosphate-buffered saline and counted. Viability was assessed by trypan blue exclusion.

Table 1. Characteristics of the patients.

Patient	Sex	Age, years	Treatment	Status
1	M	34	α-IFN + HU; ASCT	CCR[a]
2	F	39	α-IFN + HU; ASCT	CP
3	M	31	HU; allo BMT, URD	CCR
4	M	23	HU; allo BMT, URD	CCR
5	M	27	HU; allo BMT, URD	CCR
6	M	33	α-IFN; allo BMT, URD	CCR
7	M	35	α-IFN + HU; allo BMT, RD	CCR
8	M	38	α-IFN + HU; allo BMT, FD	CCR
9	F	45	α-IFN + HU; allo BMT, FD	CCR
10	M	53	α-IFN + HU; allo BMT, FD	CCR
11	F	24	α-IFN + HU; allo BMT, FD	CCR
12	F	37	α-IFN + HU; allo BMT, FD	CCR
13	M	55	α-IFN + HU; allo BMT, FD	CCR

[a] CCR, complete cytogenetic remission; CP, chronic phase; allo, allogeneic; URD, unrelated donor; RD, related donor.

Where indicated, erythrocytes were removed by lysis with NH_4Cl, and the remaining cells were washed in phosphate-buffered saline before nucleated cells were lysed. Leukapheresis samples were collected from patients who had undergone ASCT, and were stored frozen. Cells were washed once in phosphate-buffered saline before lysis in extraction buffer.

The cell line K562, carrying the *BCR-ABL* translocation, was used as a positive control, and the lymphoblastoid cell line BSM was used as a negative control.

PREPARATION OF THE OLIGO(dT)-COATED MANIFOLD SUPPORTS

The procedure has been described in detail elsewhere *(13)*. The manifold supports, shaped so that individual prongs fit the wells of a microtiter plate, were sonicated in 950 mL/L ethanol. Oligo(dT)-cellulose (Amersham Pharmacia Biotech) was rinsed repeatedly in triethylamine before being mixed with triethylamine to obtain a slurry. The manifold supports were immersed for 2 s in the slurry, washed once in ethanol and once in water, allowed to air dry, and stored at 4 °C until used.

RNA ISOLATION AND cDNA SYNTHESIS

Cells were counted and lysed in extraction buffer containing 100 mmol/L Tris-HCl (pH 7.9), 10 mmol/L EDTA, 5 mmol/L dithiothreitol, 500 mmol/L lithium chloride, and 10 g/L lithium dodecyl sulfate to a density of 10^7 cells/mL. Viscosity was reduced by repeated passage through a needle (0.7 × 50 mm). The manifold supports were presoaked in extraction buffer for 5 min and submerged in 50 μL of cell lysate. mRNA was captured by hybridization to the supports on a shaking platform for 30 min at room temperature. The supports were subsequently washed eight times in 80 μL of 10 mmol/L Tris-HCl (pH 7.5), 0.1 mol/L NaCl, and 1 mmol/L EDTA; transferred to fresh microtiter wells containing 50 μL of 50 mmol/L Tris-HCl (pH 8.3), 75 mmol/L KCl, 3 mmol/L $MgCl_2$, 10 mmol/L dithiothreitol, 0.5 mmol/L dNTPs, 0.25 g/L bovine serum albumin, 2.5 μmol/L random DNA hexamers (Pharmacia Biotech), 25 units of HPRI™ ribonuclease inhibitor (Amersham Life Science), and 200 U of M-MLV reverse transcriptase (Amersham Life Science); and incubated at 37 °C for 1 h. The cDNA was eluted from the manifold support by denaturation at 95 °C for 5 min in 50 μL of water and amplified immediately or stored at −20 °C.

PRIMERS AND PROBES

Primers and 5′-nuclease probes were designed using Primer Express (PE Biosystems) software (Table 2). Two different forward primers were selected for the *BCR-ABL* transcript, one located in exon b2 and one located in exon b3 of the *BCR* gene. The 5′-nuclease probe was located in exon 2 of the *ABL* gene. The reference *β-ACTIN* cDNA was amplified with intron-spanning primers and the probe from the TaqMan *β-ACTIN* control reagent kit

Table 2. Primer and probe sequences.[a]

	Forward primer	TacMan probe	Reverse primer
b2a2	GCATTCCGCTGACCATCAATA	FAM-CAGCGGCCAGTAGCATCTGACTTTGA-TAMRA[b]	TCCAACGAGCGGCTTCAC
b3a2	CCACTGGATTTAAGCAGAGTTCAA	FAM-CAGCGGCCAGTAGCATCTGACTTTGA-TAMRA	TCCAACGAGCGGCTTCAC
GAPDH	GAAGGTGAAGGTCGGAGTC	JOE-CAAGCTTCCCGTTCTCAGCC-TAMRA	GAAGATGGTGATGGGATTTC
BCR	TCTCCCTGACATCCGTGG	FAM-TGAAACTCCAGACTGTCCACAGCATTCC-TAMRA	GATGACATTCAGAAACCCATAGAG
β-ACTIN	CAACTGGGACGACATGGAGA	FAM-ATGCCCT^{TAMRA}CCCCCATGCCATCCTGCGT	GGTCAGGCAGCTCGTAGCTC

[a] All sequences are 5′ to 3′.

[b] FAM, 6-carboxyfluorescein; TAMRA, 6-carboxy-tetramethylrhodamine; JOE, 2,7-dimethoxy-4,5-dichloro-6-carboxyfluorescein.

(PE Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) cDNA was amplified with the TaqMan *GAPDH* control reagents kit (PE Biosystems).

PCR amplification

cDNA (10 μL) was transferred to an optical microtiter well containing 25 μL of buffer A (PE Biosystems); 2 mmol/L $MgCl_2$; 0.2 mmol/L dGTP, dATP, and dCTP; 0.4 mmol/L dUTP; 0.5 μmol/L each amplification primer for *BCR-ABL*; 0.1 μmol/L TaqMan probe; 1.25 U of AmpliTaq Gold™ (PE Biosystems); and 0.25 U of Amperase UNG™ (PE Biosystems). The reactions were incubated at 50 °C for 2 min and 95 °C for 10 min, and cycled 45 times between 95 °C for 15 s and 60 °C for 1 min in the ABI Prism 7700 (PE Biosystems). For the reference genes, PCR amplification was started with 5 μL of cDNA.

Construction of the plasmid calibrators

cDNA was prepared from a patient with a b2a2 translocation. *BCR-ABL* and *GAPDH* sequences were amplified with the described PCR primers. The amplification products were cloned into the pCRII vector (Invitrogen) and sequenced. Known amounts of each plasmid were linearized with *Hin*dIII and *Eco*RV, respectively. Serial dilutions (10-fold) representing 5 to 5×10^5 copies of pCRBCR-ABL and 4 to 4×10^5 copies of pCRGAPDH were prepared from the cleaved plasmids, aliquoted, and stored at −20 °C.

Measurement of *BCR-ABL* mRNA in patient samples

Serial dilutions from 4 to 4×10^5 copies of pCRGAPDH and 5 to 5×10^5 copies of pCRb2a2 were amplified in duplicate. A calibration curve was derived by plotting the C_T values obtained for each dilution against the logarithm of the plasmid copy number. The mean slopes of the calibration curves for the two genes were −3.42 for *GAPDH* and −3.6 for *BCR-ABL*, with interassay CVs of 6% and 4%, respectively. The calibration curves show a strong linear correlation, with correlation coefficients (r^2) between 0.97 and 0.997.

BCR-ABL and *GAPDH* sequences were amplified in duplicate from the patient samples, and the copy numbers of both genes were calculated with help of the respective calibration curves. The estimated amount of *BCR-ABL* mRNA was normalized by dividing by the amount of *GAPDH* mRNA to compensate for variations in quantity or quality of starting mRNA as well as for differences in reverse transcriptase efficiency. The normalized values were multiplied by the constant 10^4.

Because sample size and quality vary between individual samples, interpreting negative or weakly positive results may be difficult. We thus calculated the absolute limit of detection of *BCR-ABL* mRNA for each individual sample. The limit of detection was calculated by dividing the theoretically lowest number of *BCR-ABL* molecules detectable by PCR, i.e., one molecule, by the number of *GAPDH* molecules detected in that particular sample, and multiplying by 10^4.

Results

We designed an efficient and accurate assay to monitor *BCR-ABL* mRNA expression in peripheral blood from CML patients. The different steps of the assay were validated with respect to the C_T value obtained in the TaqMan assay and optimized for maximal sensitivity and reproducibility.

Reproducibility of sample preparation

To determine the variability in binding capacity of the manifold supports and in the sample preparation procedure, mRNA from cell lysates was captured on individual prongs of different manifold supports. RNA from lysates corresponding to 2×10^4 and 2×10^5 K562 cells was immobilized on 6 and 10 individual prongs, respectively. After reverse transcription, the cDNA was amplified with primers and probes specific for *GAPDH*. Fig. 1 shows the individual C_T values and the average of the C_T values for the different RNA preparations. The SD of the C_T value was 0.6 and 0.7 cycles for samples containing 2×10^4 and 2×10^5 cells, respectively.

Sample handling

Successful amplification of RNA from blood samples required the elimination of the erythrocytes before cell lysis (data not shown). Erythrocytes were removed either by Ficoll separation or by hypotonic lysis with NH_4Cl, and the remaining cells were counted in an hemocytometer before being lysed for RNA capture. Isolation of leukocytes from whole blood by Ficoll gradient centrifugation consistently gave a lower C_T value than red cell lysis with hypotonic NH_4Cl (Fig. 2). In additional exper-

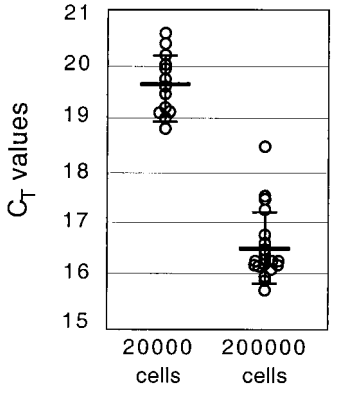

Fig. 1. Reproducibility of sample preparation.

GAPDH was amplified in duplicate from 6 separate RNA preparations corresponding to 20 000 K562 cells and from 10 separate RNA preparations corresponding to 200 000 K562 cells. The individual C_T values, as given by the software of the ABI Prism 7700 instrument, plus mean (*horizontal bar*) and SD (*error bars*) are shown.

iments, mononuclear cells were purified from blood by Ficoll separation before mRNA isolation on the manifold supports.

SENSITIVITY AND SPECIFICITY OF THE ASSAY

To estimate the sensitivity of the method, K562 cells were mixed with variable numbers of cells from the *BCR-ABL*-negative lymphoblastoid cell line BSM to give ratios ranging from 1:1 to $1:10^6$. mRNA corresponding to 500 000 cells of the different dilutions was bound to the manifold supports and processed through the different steps of the assay. In 50% of the cases, *BCR-ABL* mRNA could be quantified in cDNA prepared from samples containing 1 K562 cell in 10^5 BSM cells. In the remaining 50% of the cases, only one of the duplicate samples of this dilution was positive for the dilution containing 1 K562 cell in 10^5 BSM cells. Both primer combinations (b2a2 and b3a2) gave the same detection limit (1 K562 cell in 10^5 BSM cells), and the slopes of the curves were very similar (Fig. 3). These results indicate that it is possible to use one primer/probe set to amplify either fusion variant.

No *BCR-ABL* products were obtained from cDNA prepared from BSM cells or mononuclear cells from healthy individuals or when reverse transcription was omitted.

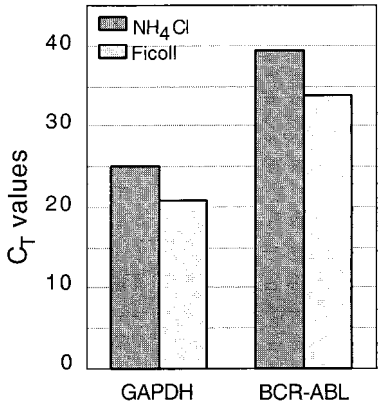

Fig. 2. Effect of sample handling procedure on the amplification of *BCR-ABL* and *GAPDH* mRNAs.

Erythrocytes were removed by lysis with hypotonic NH_4Cl or by Ficoll density centrifugation. The average C_T values from two peripheral blood samples are shown.

CHOICE OF REFERENCE GENE

Three reference genes were investigated, *β-ACTIN*, *GAPDH*, and *BCR* in samples from 12 different individuals and two cell lines to determine which gene showed the smallest variability among samples and where genomic DNA contributed the least to the signal. All samples were processed in duplicate, and the reverse transcription enzyme was omitted in every second sample. All three genes could be amplified in the absence of the reverse transcription reaction. The C_T values were on average 11 cycles higher in the case of *β-ACTIN* and *GAPDH* and 8 cycles in the case of *BCR*, compared with the corresponding samples treated with reverse transcriptase (Fig. 4). Variations in reference gene expression among different individuals were very similar for the three genes tested, as shown by the similar SD values. In subsequent experiments, *GAPDH* was used as a reference gene.

DAY-TO-DAY IMPRECISION

The overall reproducibility of the quantification assay was estimated by analyzing six different samples, starting from the RNA isolation, on four different occasions. The expression of *BCR-ABL* and *GAPDH* mRNAs was calculated by comparison with their respective calibration curves, and the normalized *BCR-ABL* values were calculated. Fig. 5 shows the means and SDs for the different samples. The mean CV for the *BCR-ABL*/*GAPDH* ratio was 32% (range, 20–44%).

ANALYSIS OF TRANSCRIPT EXPRESSION IN PATIENT SAMPLES

Lysates (50 μL) from leukocytes obtained from patient blood samples were incubated with the manifold sup-

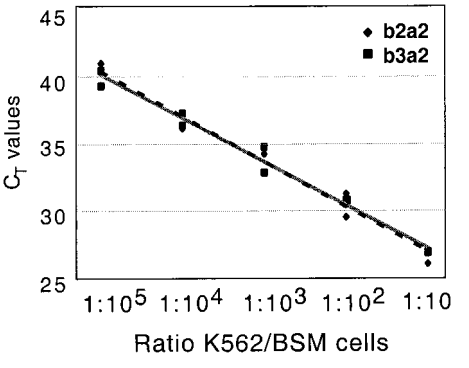

Fig. 3. Assay detection limit and primer set comparison.

K562 cells were mixed with the *BCR-ABL*-negative cell line BSM in different ratios. The cDNAs prepared from the individual mixes were amplified in duplicate with two different sets of primers, b2a2 and b3a2. The C_T values were plotted against the different dilutions of K562 cells.

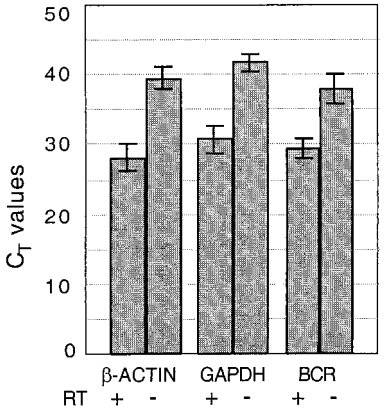

Fig. 4. Comparison of reference genes.

Analysis of equal amounts of cell lysates from 12 individuals plus two cell lines for *β-ACTIN*, *GAPDH*, and *BCR* sequences. The means of the C_Ts ± SD with (+) and without (−) the reverse transcription step are shown. *RT*, reverse transcriptase.

ports and processed as described in *Materials and Methods*. Fig. 6 shows the *BCR-ABL* mRNA expression normalized to *GAPDH* mRNA expression for two different CML patients who received α-interferon (α-IFN) + hydroxyurea (HU) followed by ASCT. *BCR-ABL* mRNA expression in patient 1 decreased from 22 arbitrary units to below the detection limit after ASCT. The patient remained PCR-negative for 1 year after ASCT, with one transitional PCR-positive analysis in the 6 months follow-up sample. In an analysis 90 months after ASCT, *BCR-ABL* expression had increased to ~25 arbitrary units, although the patient remained in clinical and cytogenetic

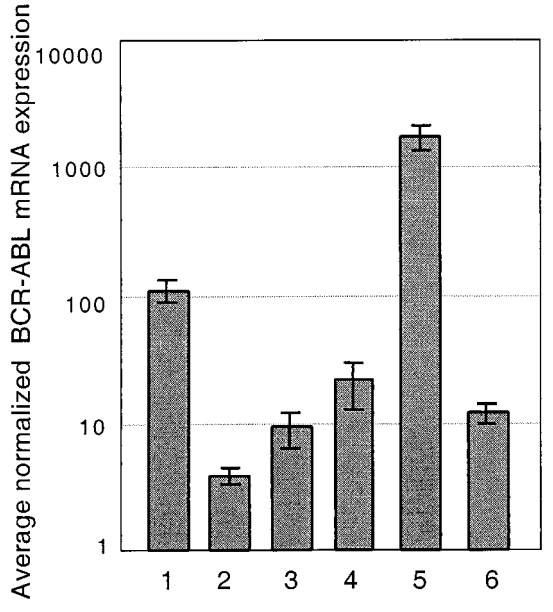

Fig. 5. Overall assay variability.

Quantification of *BCR-ABL* mRNA expression in six different patient samples in four independent experiments. The amount of *BCR-ABL* mRNA is expressed in arbitrary units obtained by dividing the *BCR-ABL* copy number by the *GAPDH* copy number and multiplying by 10^4. The means ± SD (*bars*) for the normalized *BCR-ABL* expression are shown.

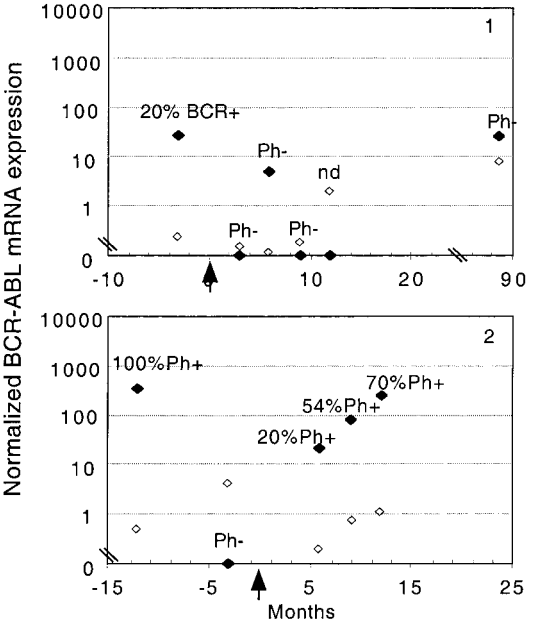

Fig. 6. *BCR-ABL* mRNA expression in patients receiving ASCT.

BCR-ABL mRNA expression was quantified in two patients before and after ASCT. The amount of *BCR-ABL* mRNA is expressed in arbitrary units obtained by dividing the *BCR-ABL* copy number by the *GAPDH* copy number and multiplying by 10^4. Normalized *BCR-ABL* mRNA expression is shown over time (expressed in months). *Arrows* indicate ASCT. ♦, normalized *BCR-ABL* expression; ◇, absolute detection limit for a particular sample. Results from the cytogenetic analysis are indicated as percentage of Philadelphia-positive (*Ph+*) cells in bone marrow smears. *Ph−*, no Philadelphia-positive metaphases.

remission. Patient 2 did not respond clinically to ASCT. The increases in both normalized *BCR-ABL* mRNA expression and numbers of Philadelphia-positive cells also reflect this fact.

The rest of the patients received allogeneic BMT, patients 3–6 from an unrelated donor and patients 7–13 from a relative. In this group of patients, a pretransplantation sample was available for patients 4, 5, and 6. The normalized *BCR-ABL* mRNA expression at diagnosis was on average 805 arbitrary units (range, 230–3200; Fig. 7). After BMT, *BCR-ABL* mRNA expression dropped dramatically, between 1000- and 10 000-fold, over a period of 3–6 months and became undetectable in patient 5, 3 months after BMT (Fig. 7), and in patient 6, 9 months after BMT (Fig. 7). In three additional patients analyzed 30–48 months after BMT, the normalized *BCR-ABL* mRNA expression was below the detection limit (data not shown). After allogeneic BMT (6–12 months), *BCR-ABL* mRNA expression was always below 5 arbitrary units. The patients receiving allogeneic BMT remained in hematological and cytogenetic remission during the period studied, except patients 7 and 10. Both patients were transiently Philadelphia chromosome positive (1 cell in 50) in a bone marrow sample 12 and 24 months after BMT, respectively.

Discussion

In the present report, we describe a reliable and sensitive method to monitor patients with CML by measuring

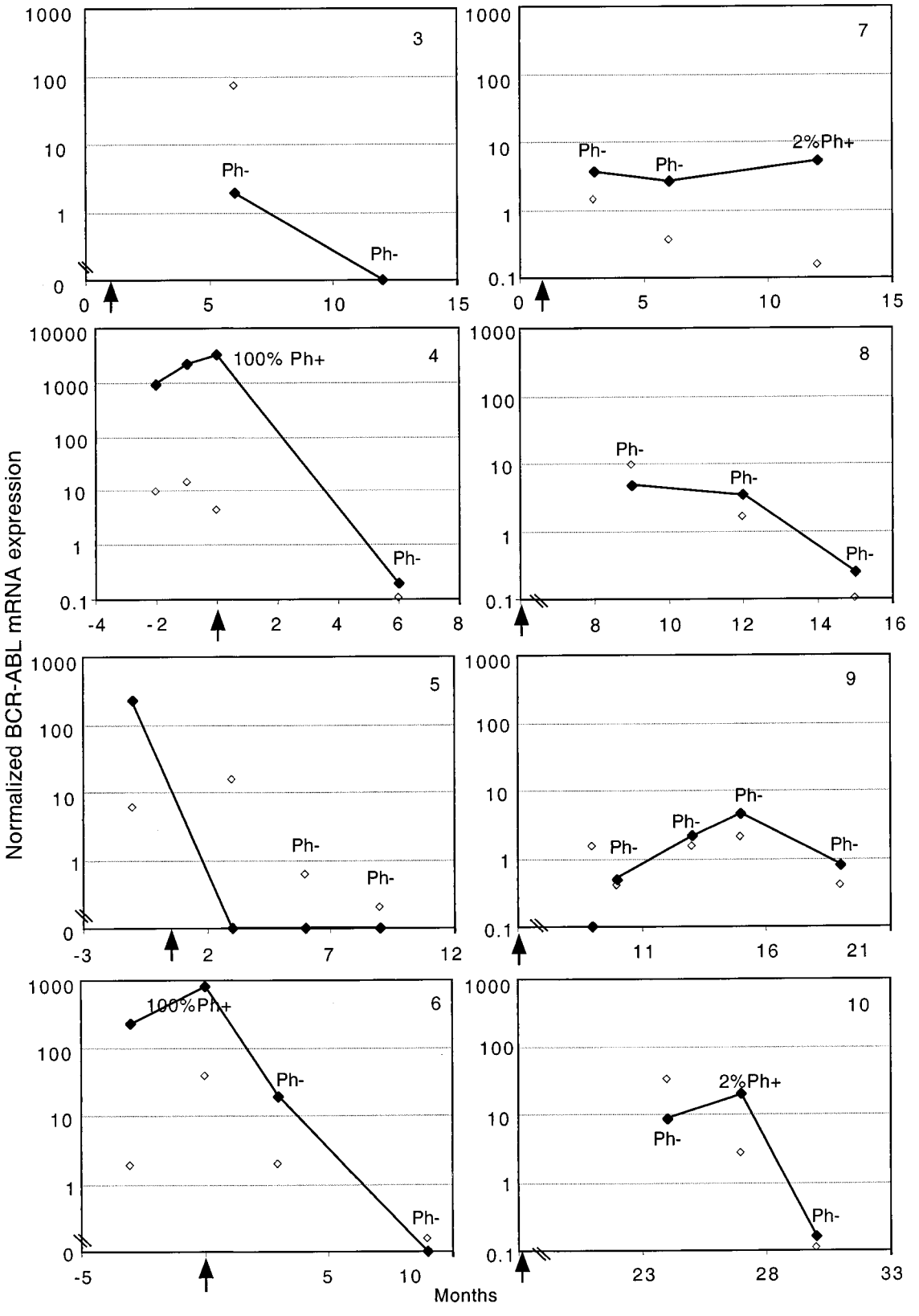

Fig. 7. *BCR-ABL* mRNA expression in patients receiving BMT.

BCR-ABL mRNA expression was quantified in eight patients who had received allogeneic BMT. Normalized *BCR-ABL* expression is shown over time (expressed in months). *Arrows* indicate allogeneic BMT. ◆, normalized *BCR-ABL* expression; ◇, absolute detection limit for each individual sample; *Ph−*, no Philadelphia-positive metaphases. Results from the cytogenetic analysis are indicated as the percentage of Philadelphia-positive (*Ph+*) cells in bone marrow smears.

BCR-ABL mRNA in peripheral blood. The method is suitable to follow CML patients after BMT.

We propose a streamlined RNA isolation method where poly(A)+ mRNA is isolated directly from the cell lysate by binding to an oligo(dT)-coated manifold support. Manifold supports are devices composed of sets of prongs that project into a corresponding set of reaction wells. The surfaces of the prongs may be modified to allow the binding of biomolecules so that these can be processed in a set of reactions and loaded on detection instruments.

The use of manifold supports in molecular diagnostics has been useful for genotyping by the oligonucleotide ligation assay or minisequencing, and for the detection of mutations by sequencing [reviewed in Ref. *(14)*]. Because the RNA binds to a solid support, multiple samples can be manipulated in parallel with minimal effort. Moreover, the reduction in the number of sample processing and pipetting steps diminishes the risk for uncontrolled variables associated with the quality of the RNA. Currently, instability of RNA limits the use of RT-PCR to research laboratories with trained personnel. The use of manifold supports greatly simplifies mRNA isolation, making RT-PCR-based analysis more accessible to routine laboratories. The method can also be adapted to other oligo(dT)-coated solid supports, such as paramagnetic beads. We found that 25 μL of Dynabeads (Dynal) had a binding capacity similar to that of individual prongs in our manifold support (data not shown).

Both *β-ACTIN* and *GAPDH* are known to have pseudogenes that can give rise to a PCR product that interferes with the quantification of these RNAs. The presence of genomic DNA in the RNA preparation thus represents a potential source of error when quantifying the reference gene. The difference in C_T values (11 cycles) that we found between the samples amplified with and without reverse transcriptase shows that genomic DNA contamination of the mRNA bound to the manifold support is negligible.

We found that a single primer/probe set was adequate to amplify the two most common *BCR-ABL* fusion variants, which simplifies the assay and eliminates the need to analyze the fusion variant of individual patients. This result is in agreement with two recent reports where all fusion variants of *BCR-ABL* could be quantified using a single set of primers *(15, 16)*.

We also examined how sample preparation affected the sensitivity of the assay. It was necessary to remove erythrocytes to obtain a positive PCR, probably because of the known inhibitory effect of hemoglobin *(17)*. Peripheral blood mononuclear cell purification by Ficoll density separation gave lower C_T values for both *BCR-ABL* and *GAPDH* mRNAs, presumably because of better RNA quality.

Because clinical specimens are collected remote from the site of analysis, differences in time from sample collection to RNA isolation may lead to variable degradation of RNA in the sample, with variability in assay sensitivity as a consequence. We calculated the absolute detection limit of every sample, defined as one single copy of *BCR-ABL* divided by the number of copies of *GAPDH* for a particular sample, multiplied by the constant 10^4. This value should constitute a valuable help in interpreting results by reflecting the size and quality of the sample. The absence of *BCR-ABL* amplification can only be interpreted as a true negative when amplification of the reference gene reveals a positive result. In addition, positive samples where the normalized *BCR-ABL* mRNA expression falls below the detection limit are to be interpreted with caution.

The interassay imprecision (CV) was low (4–6%) for calibrators but high for patient samples (mean, 32%). This implies that the precision of the present method is insufficient to analyze small differences in *BCR-ABL* mRNA expression between samples.

The normalized *BCR-ABL* expression measurements from 13 CML patients correlated well with clinical and cytogenetic data. Recently, several groups have reported analysis of CML patients using real-time RT-PCR *(15, 18–20)* and found that real-time PCR was suitable for following the kinetics of *BCR-ABL* mRNA in CML. The sensitivity of our method is comparable to that of Preudhomme et al. *(20)* and Eder et al. *(19)*, indicating that the manifold support has sufficient capacity to monitor minimal residual disease, where only a small minority of the cells are positive for *BCR-ABL* fusion mRNA.

Typically, the normalized *BCR-ABL* values for untreated patients were between 250 and 1000 arbitrary units. Patients that show a partial response (α-IFN + HU and ASCT) had *BCR-ABL* values between 100 and 10 arbitrary units. After allogeneic BMT, *BCR-ABL* mRNA fell dramatically to <5 arbitrary units. Additional studies will be necessary to determine what threshold *BCR-ABL* mRNA expression is critical and predictive of relapse.

In conclusion, the combination of a manifold support to isolate mRNA in a fast and reproducible manner, together with the advantages of real-time RT-PCR is promising as a sensitive and accurate method to study the kinetics of *BCR-ABL* mRNA expression, and is suitable to monitor minimal residual disease in CML patients.

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