Preclinical Validation of a Monochrome Real-Time Multiplex Assay for Translocations in Childhood Acute Lymphoblastic Leukemia

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ABSTRACT

Purpose: **The purpose is to develop a real-time multiplex reverse transcription-PCR assay for detection and quantification of leukemia-specific chimeric transcripts that identify the genetic subgroups of acute lymphoblastic leukemias (ALLs) proposed by the WHO classification.**

Experimental Design: **Real-time multiplex assay for t(12;21), t(4;11), and t(1;19) with hypoxanthine phosphoribosyltransferase as internal standard used in tandem with a new real time quantitative-RT-PCR assay for the t(9;22). This new strategy was designed to yield an amplicon from each translocation with a distinct melting peak allowing dependable identification using only Sybr green I, without any need for expensive hybridization probes.**

Results: **We validated this method with 92 primary ALLs and identified 4 *E2A-PBX*1, 4 m*BCR-ABL* and 10 *TEL-AML1*. When compared with conventional RT-PCRs and Southern blot analyses, 100% concordance was obtained. During the course of these studies, we found marked variations in the levels of the *TEL-AML1* transcripts in individual patients. We, therefore, extended the study to accurately and reproducibly determine *TEL-AML1* mRNA levels in 47 additional patients with t(12;21). The results indicated that the level of expression of *TEL-AML1* varied among individual patients, and it was independent of the WBC count.**

Conclusions: **Our new real-time multiplex assay can be used for rapid, simple, and reliable classification of pediatric ALL. Its reproducible quantification results should also facilitate studies on minimal residual disease. The observed variation in *TEL-AML*1 transcript levels is of interest because it could reflect biological and/or clinical heterogeneity in the behavior of these leukemias.**

INTRODUCTION

Childhood precursor B cell ALLs[2] frequently carry specific chromosomal translocations (1–3). Although >50 distinct rearrangements have been described, four, *i.e.*, t(12;21), t(1;19), t(9;22), and t(4;11), constitute the most frequently observed abnormalities.

These recurrent chromosomal translocations, which typically lead to the formation of fusion genes and their expression as chimeric transcripts and proteins (4), are of relevance in leukemogenesis because they influence the expression of key regulatory genes in the hematopoietic system. It is now generally accepted that these four chromosomal translocations have therapeutic importance for risk assessment in current multiagent chemotherapy protocols because they are predictors of prognosis (5–8).

The identification of these four translocations is thus important not only for understanding the biology of ALL but also as a guide for patient management. The recognition of this has allowed precursor B ALL to be subdivided into molecular entities in the recently proposed WHO classification (9). The presence of t(12;21) is associated with good response and detected in 20–25% of pediatric ALL (10). The t(1;19) is observed in 5–6% of all ALLs but constitutes 25% of the pre-B subgroup and predicts intermediate response (11). The other two translocations, t(9;22) and t(4;11), are each observed in 5–6%, and both associate with poor response but the latter occurs mostly in infant patients (12, 13).

Fluorescence *in situ* hybridization, Southern blot, or RT-PCR strategies can successfully detect these translocations. However, all these methods are sample/time consuming and are not accurate for quantification purposes. Except for RT-PCR, they are directed toward one specific rearrangement at a time. More recently, real-time amplification assays have been developed and they have several advantages (14). Reactions are characterized by fluorescence appearance during the exponential phase of amplification when none of the reagents is limiting and, therefore, allowing a more precise quantification. Because this is based on fluorescence detection allows analysis of minimal amounts of template with high sensitivity. The real-time system also obviates post-PCR manipulation preventing car-

Received 4/8/02; revised 7/17/02; accepted 7/22/02.
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[2] The abbreviations used are: ALL, acute lymphoblastic leukemia; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; PBL, peripheral blood lymphocyte; *HPRT*, hypoxanthine phosphoribosyltransferase; RT-PCR, reverse transcription-PCR.

Table 1 Primers and major amplicons' identities

Amplicon	Primer F	Primer R	Size[a] (bp)	Melting peak[a] (°C)
TEL-AML1	aagcccatcaacctctctcatc (*TEL*, exon 5)	tggaaggcggcgtgaagc (*AML1*, exon 3)	181	86 ± 0.5
*E2A-PBX*1	gccacggggcgctggcctcaggttt (*E2A*, exon 12)	cacgccttccgctaacag (*PBX*1, exon 2)	275	93.9 ± 0.4
MLL-*AF4*	agaatcaggtccagagcagagc (MLL, exons 8 to 9)	atgctgagagtcctttgtaggg (*AF4*, exons 5 to 6)	358	83.6 ± 0.3
m*BCR-ABL*	acctcacctccagcgaggaggactt (*BCR*, exon 1)	tccactggccacaaaatcatacagt (*ABL*, exon 2)	418	92.2 ± 0.3
HPRT	gttggatataagccagactttgttg	actcaacttgaactctcatcttaggc	164	80.8 ± 0.3
GAPDH	cgggaagcttgtcatcaatgg	catggttcacacccatgacg	221	89.1 ± 0.3

[a] For the major transcript.

ryover contaminations, which is critical in a clinical setting. Although recent reports have dealt with real-time PCR for *BCR-ABL* (15), *E2A-PBX*1 (16), and *TEL-AML1* (17, 18), they are single PCRs based on the Taqman technology that requires the use of dual-labeled fluorogenic-specific oligonucleotides. None of the reported assays is designed to interrogate multiple translocations in a single reaction.

Consequently, our aim was to develop a real-time multiplex strategy to determine the presence of the chimera transcripts, which will also allow quantification.

We report here a simple, robust, and reproducible new multiplex system based on real-time RT-PCR to detect all of the four translocations necessary for the identification of the major genetic subgroups of ALL. The additional advantage is that it is a monochrome assay that does not require hybridization probes and is hence less expensive. We also show that the system is highly precise for quantification purposes using the *TEL-AML1* transcript as an example. This analysis further demonstrated a notable interpatient variation in the expression level of *TEL-AML1*, which could not be accounted for by the leukemia burden. Because this variation appears to be an intrinsic factor, it may influence the biology of this ALL and, consequently, have clinical significance.

MATERIALS AND METHODS

Biological Samples. We have used the following leukemia cell lines that carry chromosomal translocations to develop the multiplex assay: REH with the t(12;21)(p13;q22); 697 with the t(1;19)(q23;p13); SupB15 with the t(9;22)(q34;q11); and RS4;11 with the t(4;11)(q21;q23). Cells were grown at 37°C in a 5% CO_2 atmosphere in RPMI 1640 supplemented with 2 mM glutamine and 10% fetal bovine serum, containing also 100 units/ml penicillin and 0.1 mg/ml streptomycin.

Samples from 92 children with primary precursor B cell ALL were also analyzed. The median age of the patients was 5 years (range, 0 to 14 years). Bone marrow aspirates or peripheral blood samples were collected at the time of routine diagnostic procedures. Written informed consent was obtained from the guardians under approved Institutional Review Board projects. Fifty-two samples were collected from the All India Institute of Medical Sciences (New Delhi, India), and 40 were collected from the King Fahad National Centre for Children's Cancer and Research, King Faisal Specialist Hospital and Research Centre (Riyadh, Saudi Arabia). Forty-seven additional ALLs carrying a 12;21 translocation were available from Istanbul University (Istanbul, Turkey) and were specifically analyzed to evaluate the level of *TEL-AML1* expression.

RNA Extraction and cDNA Synthesis. Patients' mononuclear cells were obtained from ficoll-hypaque density gradient centrifugation and lyzed with Trizol (Invitrogen Life Technologies, Inc., Carlsbad, CA) for RNA extraction. The quality of RNA was checked by absorbance at 260 and 280 nm and gel electrophoresis. Total RNA (2–3 μg) from patient samples or cultured cell lines was reverse transcribed using the Superscript first strand system for RT-PCR (Invitrogen, Carlsbad, CA) and random hexamers.

End Point RT-PCR for the Four most Frequent Translocations. The quality and integrity of cDNAs were tested by initially amplifying the housekeeping gene *GAPDH*. All samples were then analyzed by standard RT-PCR reactions for each of the four rearrangements. The primers used are shown in Table 1.

Once the RT-PCR reactions were standardized, a multiplex RT-PCR reaction was developed. In a 50-μl reaction, all four sets of primers were included at 30–100 pmols and 1.5 mM $MgCl_2$ in a program of 4 min at 95°C, 35 cycles of 30 s at 95°C, 30 s at 56°C, 1 min at 72°C, and finally 10 min at 72°C. Each amplicon was designed to contain the specific junction giving a different size band after 4% agarose gel electrophoresis (Table 1). This easy identification of the translocation present was used to test all patient samples.

Multiplex Real-Time RT-PCR. The real-time assay was based on the Lightcycler instrument (Roche, Mannheim, Germany) using fluorogenic Sybr Green I. The PCR reactions were carried out in 20 μl, including 10–15 pmols of each primer, 2.5 mM $MgCl_2$ and reagents from the FastStart DNA master Sybr green I kit (Roche). The program consisted of 10 min at 95°C followed by a touchdown setting of 32 cycles of 10 s at 95°C, 5 s at 62–58°C and 10 s at 72°C and a final cycle of 20 s at 97°C, 15 s at 65°C, 0 s at 98°C, and cooling to 40°C.

All experiments were conducted multiple times (at least three) using the appropriate positive, negative, and no template

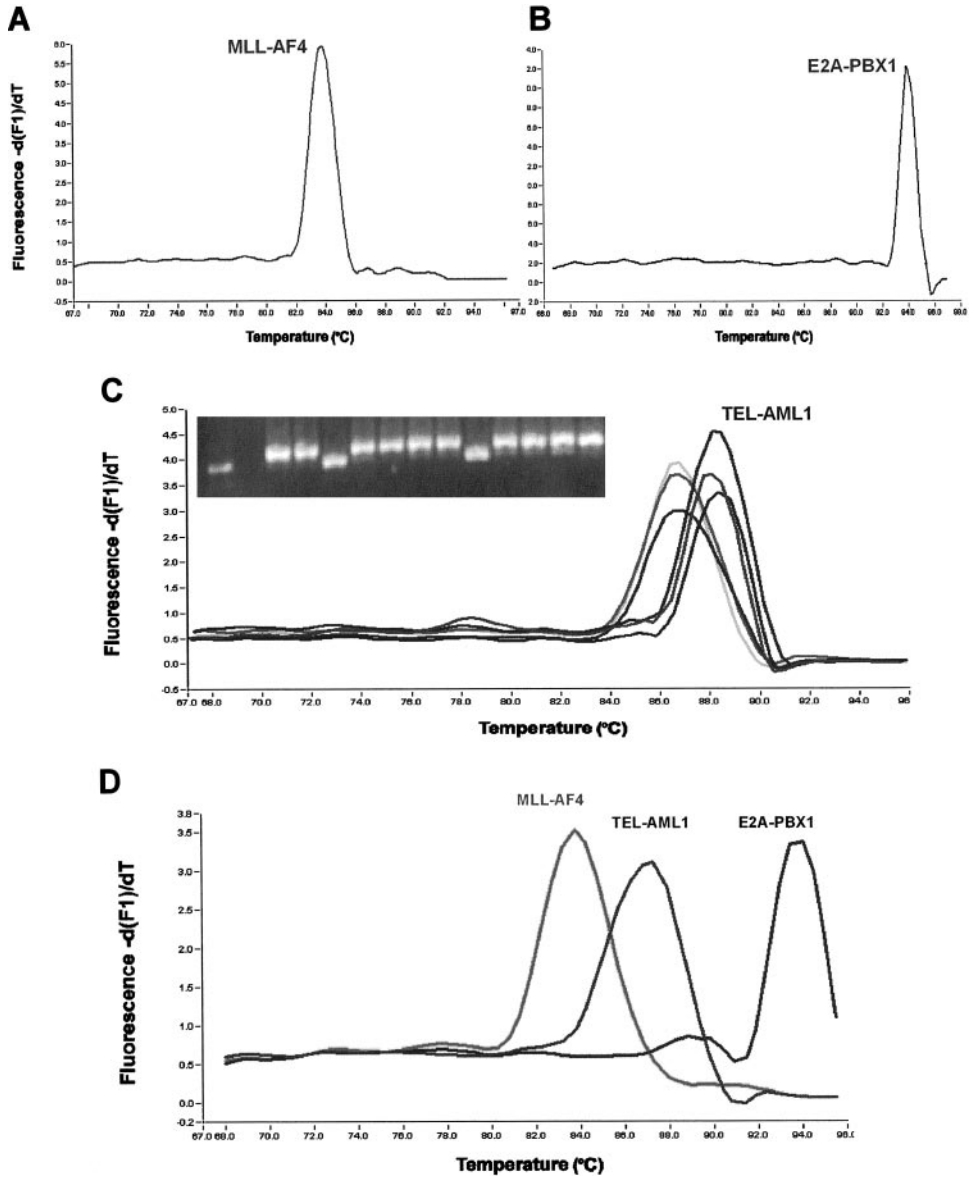

Fig. 1 Real-time RT-PCR for three translocations using cDNA from positive control cell lines. Melting peaks are shown for t(4;11) with Tm = 83.6 ± 0.3°C (*A*), t(1;19) with Tm = 93.9 ± 0.4°C (*B*), and t(12;21) with Tm = 86 ± 0.5°C for the most frequent breakpoint in intron 2 and higher Tm (88°C) for the variant, smaller isoform (*C*). The *inserted photograph* of an ethidium bromide stained gel shows the different sizes of the amplicons according to the breakpoint; three samples carry the variant isoform resulting from intron 3 breakpoints. The fidelity of the melting peaks in a multiplex reaction is shown in *D*.

controls. Fluorescence emission spectra were monitored in real time for amplification kinetics, and melting curve analyses were performed to assess the specificity of the amplified products.

Quantitative Real-Time RT-PCR. PCR products were measured by the threshold cycle (or crossing point, Cp) at which fluorescence became detectable above the baseline. The Cp was used for kinetic analysis and was proportional to the initial number of target copies in the sample.

We calculated the relative expression of *TEL-AML1* transcripts as the ratio between the level of *TEL-AML1* and the level of *GAPDH*. For this purpose, we generated standard curves by measuring the Cp values for *GAPDH* transcripts from serial dilutions of REH cDNA. The *TEL-AML1* amplicon was cloned in a TA vector (Invitrogen), and serial 10-fold dilutions of this plasmid, corresponding to known copy numbers, were run in triplicates in the Lightcycler. For statistical correlations with clinical features, the Pearson χ^2 test and the Kaplan-Meier analysis were used.

Southern Blot Analyses. Ten μg of genomic DNA from patient samples, PBLs from normal individuals and control cell lines, REH and RS4;11, were independently digested with *Bam*HI and *Hin*dIII. Restricted fragments were separated in a 0.8% agarose gel and transferred to nylon membranes. Blots were hybridized with ^{32}P-radiolabelled *TEL* and *MLL* probes (19).

RESULTS

Identification of Translocations by Real-Time RT-PCR. We initially established conventional end point RT-PCR reactions individually for the four most common translocations in childhood ALL that define clinical subgroups (3, 9). Primers were chosen to amplify all of the breakpoints and splice variants

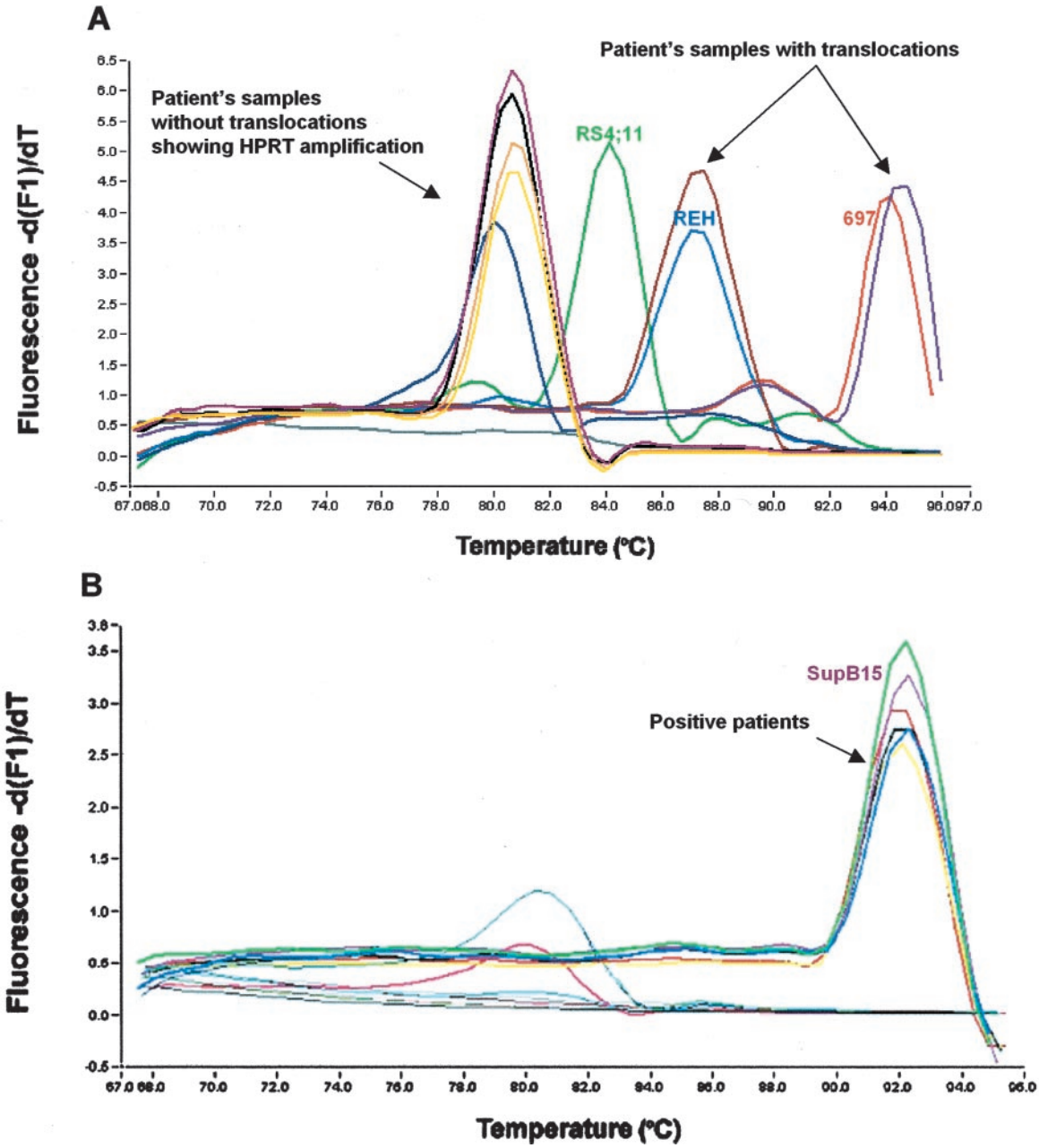

Fig. 2 Validation of the assay using ALL patient samples. Positive control cell lines were included in each run, and the signals obtained are indicated. *A*, real-time multiplex RT-PCR, including the internal standard. Samples with none of these three translocations show amplification of *HPRT* and samples with t(12;21) or t(1;19) show the respective peak. *B*, tandem reaction for m*BCR-ABL*. Positive samples clearly show the characteristic peak. We have specifically included a small fraction of negative patients that demonstrate nonspecific, low signals.

known (20), except for infrequent MLL-*AF4* rearrangements (<15%) and an extremely rare *E2A-PBX*1 chimera (21). These specific primers were designed to give amplicons of different sizes (Table 1), allowing us to subsequently combine them in a single multiplex reaction and identify them by the size of the band in 4% agarose gels. The identity of each amplicon was additionally confirmed by hybridization to internal oligonucleotide probes (data not shown).

We then translated the individual amplifications to the Lightcyler system (Roche) using Sybr Green I. We had determined a $MgCl_2$ concentration of 2.5 mM as optimum for all of the four reactions. To successfully multiplex the system, we screened for conditions that would diminish primer-dimer formation and the signals resulting from them. To achieve this, we standardized the assays using a temperature release *Taq*DNA polymerase and a temperature touchdown amplification program consisting of 32 cycles. As shown in Fig. 1, *A–C* and Table 1, each amplicon yielded a unique, characteristic, and reproduc-

Table 2 Clinical characteristics of the patients carrying translocations

Patient	Sex	Age (yr)	WBC ($\times 10^9$/liter)	Fusion gene
1	M	3	13.5	*TEL-AML1*
2	M	5	4.26	*TEL-AML1*
3	M	3	3.11	*TEL-AML1*
4	F	2	31	*TEL-AML1*
5	M	6	4.93	*TEL-AML1*
6	M	5	5.92	*TEL-AML1*
7	M	2	11.7	*TEL-AML1*
8	F	4	5.67	*TEL-AML1*
9	M	6	27.3	*TEL-AML1*
10	M	6	6.7	*TEL-AML1*
11	M	3	400	*BCR-ABL*
12	M	8	21.3	*BCR-ABL*
13	M	9	127	*BCR-ABL*
14	M	11	120	*BCR-ABL*
15	F	7	132	*E2A-PBX*1
16	M	4	181	*E2A-PBX*1
17	M	9	66.7	*E2A-PBX*1
18	M	11	254	*E2A-PBX*1

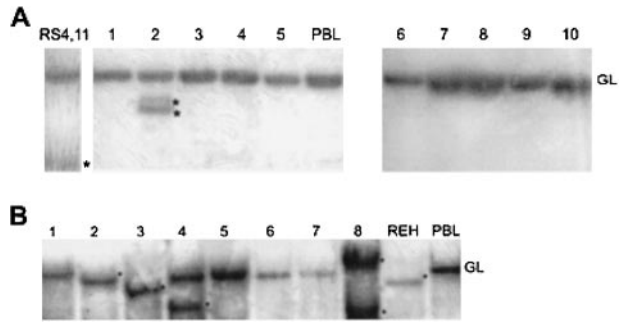

Fig. 3 Southern blot analysis of *Bam*HI digested DNAs. *A*, hybridization of two blots with an MLL probe. PBLs from a healthy control were used as a normal, germ-line (GL) control. RS4;11 demonstrates a rearranged band (∗) because of a 4;11 translocation. *Lane 2* shows rearrangement of the MLL gene (∗). *B*, another blot was hybridized with a *TEL* probe. PBL shows the GL band and REH shows a rearranged band (∗). *Lanes 2*, *3*, *4*, and *8* show rearranged bands indicated with ∗, demonstrating t(12;21).

Table 3 Distribution of the four translocations in precursor B cell ALL from non-Western countries

	TEL-AML1	*E2A-PBX*1	m*BCR-ABL*	MLL-*AF4*	None/ other
Saudi Arabia ($n = 40$)	8	0	0	0	32[a]
India ($n = 52$)	2	4	4	0	42

[a] One patient demonstrated an 11q rearrangement by Southern blot analysis using a MLL probe.

ible melting profile detected only from amplification of the corresponding positive cell line cDNA. Negative control RNAs consistently demonstrated absence of any signals, indicating the absence of false positives under these conditions.

The specificity of the melting peaks is of course determined, in part, by the nucleotide sequence of the amplicons. This is especially important in those cases where alternative splicing has been reported. As an example, the variant isoforms of *TEL-AML1* transcripts could be distinguished (Fig. 1*C*) demonstrating that the Sybr green I assay was specific enough to identify these alternative transcripts.

Multiplex Real-Time RT-PCR. The next step was to combine the individual assays in a multiplex reaction. We approached this by testing the translocations in different pairs and then combining them by gradually increasing the number of primer sets included in the reaction. Fig. 1*D* shows that three fusion transcripts could be readily detected and identified in a multiplex reaction maintaining the specificity and reproducibility of each melting peak. It is noteworthy that the temperature profile of each PCR product was not altered in the multiplex reaction. However, the amplicon for the m*BCR-ABL* chimera was always compromised in multiple combinations and thus, this reaction was developed separately as a tandem assay (Fig. 2*B*).

Application of the Tandem Real-Time RT-PCR to Primary Leukemias. We have tested and validated the assay with bone marrow aspirates or peripheral blood samples from 92 pediatric patients with precursor B cell ALL (Fig. 2). This study was conducted blindly, without previous knowledge of the presence of translocations in the samples.

Although cDNAs have been initially screened for *GAPDH* expression as a quality control, it was important to include an internal control (housekeeping gene) in the multiplex reaction to ensure PCR ability for all cDNA templates. We have hence included *GAPDH* primers in the multiplex reaction, but this addition repeatedly compromised amplification of two chimeric transcripts, *TEL-AML1* and MLL-*AF4*. Two other housekeeping genes, phospholipase A2 and β-actin, were similarly compromised. Three other genes, TATA box binding protein, α-tubulin, and β2-microglobulin, were properly amplified in the combined reaction, but the melting peak of the amplicon was very similar to the peak for the MLL-*AF4* PCR product interfering with interpretation of results. Finally, one gene, *HPRT* 1, could be established in the multiplex reaction and yielded an amplicon with a different melting peak (Table 1, Fig. 2*A*).

The PCR conditions were optimized to minimize primer-dimers when using clinical samples. We determined that 32 cycles of amplification produced a signal from each sample, coming either from a fusion transcript (if present) or from the housekeeping gene *HPRT*, without any background. This observation was additionally confirmed by agarose gel electrophoresis.

The use of the multiplex tandem real-time RT-PCR allowed us to also report differences in the distribution of translocations in two different non-Western ethnic groups (Saudi Arabia, $n = 40$ and India, $n = 52$). The clinical characteristics of the patients that carried translocations are depicted in Table 2. It appears that although *E2A-PBX*1 and *BCR-ABL* ALLs are underrepresented in Saudi Arabia (0 of 40), *TEL-AML1* ALL is rarely detected in India (Table 3).

Comparison of Data from Real-Time Multiplex and Standard Methods. Real-time RT-PCR results were correlated with previously established standard methods. As mentioned before, all patient samples were blindly screened by conventional, end point RT-PCR for each of the translocations. The concordance was 100%. Those samples from which DNA was available were additionally analyzed by Southern blot.

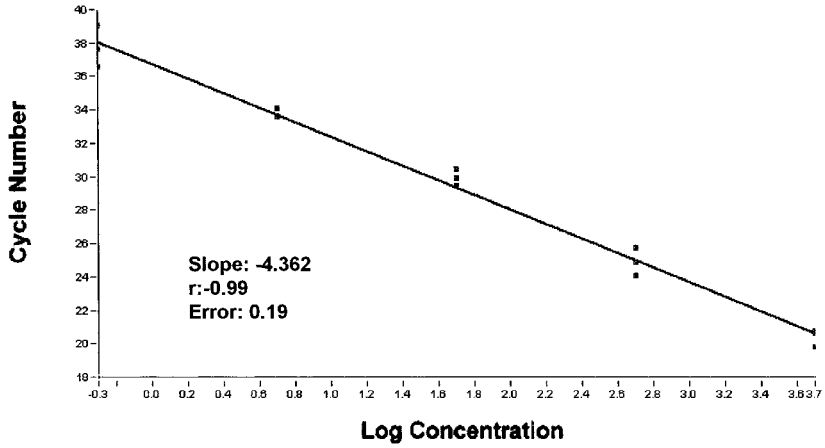

Fig. 4 Standard curve for *GAPDH* amplification. Concentration was calculated as number of cells estimating 5 pg of total RNA/cell. Five 10-fold dilutions were run in triplicate. The Lightcyler software for quantification calculated the parameters of the regression curve shown.

t(12;21) and 11q rearrangements can be identified by restriction digestion of genomic DNA followed by hybridization with *TEL* and MLL probes, respectively. One of 16 samples analyzed in *Bam*HI and *Hin*dIII digests demonstrated a rearranged MLL band (Fig. 3*A*). We then further reconfirmed that this 11q rearrangement was not because of a 4;11 translocation by performing reverse transcription of RNA with a specific *AF4* primer and amplification with three different sets of primers. Although this would have detected any of the known 4;11 breakpoints, no product was obtained from the sample. Four of the DNAs demonstrated a rearranged band when hybridized with the *TEL* probe, indicating the presence of t(12;21; Fig. 3*B*) consistent with the RT-PCR data. Therefore, in these studied cases there was an exact concordance between Southern blot and real-time multiplex data.

Quantitative Analysis of *TEL-AML1* Transcripts. Dur ing the qualitative analysis of samples for the presence of translocations, it appeared from the kinetics of the reactions that the levels of expression of the *TEL-AML1* fusion transcript varied between patients. To appropriately expand this analysis, we developed assays to specifically quantify the leukemia-specific transcript levels in relation to *GAPDH* expression as the endogenous RNA control. Each *TEL-AML1*-positive sample was independently analyzed for the levels of these two transcripts.

A real-time RT-PCR for *GAPDH* was established with a characteristic melting profile (89.1 $\pm$ 0.3°C). No genomic DNA amplification was observed as confirmed by the single melting peak detected and the absence of the DNA band (440 bp) after running the products in an agarose gel (data not shown).

The standard curve displays the linear relation between the Cp and the logarithm of the template concentration. To generate a calibration curve for *GAPDH* transcripts, five independent preparations of cDNA from the REH cell line were pooled, purified, and serially diluted in water (10-fold). Each RNA concentration was extrapolated to indicate number of cells considering 5 pg of total RNA/cell. Each data point was collected in triplicate (Fig. 4).

We cloned the *TEL-AML1* amplicon in a TA vector (pCR2.1-*TEL-AML1*) and used known amounts of this plasmid to generate a standard curve for the fusion template, measured as copy number. Ten-fold dilutions of plasmid DNA in water were made to represent values in the range of those previously observed from patients, which for this calibrator represented 4.6×10^2 to 4.6×10^7 copies. Data were obtained from triplicates with $r = 0.98$ and error $= 0.226$. This standard curve (Fig. 5) indicates a linear detection of the *TEL-AML1* transcript over at least five logs.

We calculated the interexperiment and intraexperiment variability using five independent preparations of REH cDNA and their corresponding dilutions run in two experiments in the Lightcycler for *TEL-AML1* and *GAPDH*. *TEL-AML1* transcript values ranged from 50 to 101 when normalized to *GAPDH*. Interexperiment variability was higher than intraexperiment variability, however, these variations were at the most 2-fold.

Having established the parameters of *TEL-AML1* quantification, we analyzed 47 additional cDNAs from Turkish patients carrying the 12;21 translocation in two independent experiments. Four of these patients expressed the smaller fusion (at exon 3 of *AML1*). We calculated the number of *TEL-AML1* transcripts/cell from this cohort and observed that the level of expression of *TEL-AML1* varied widely from 0.4 to 1429 with a median of 198 (Fig. 6*A*). Obviously, these differences could not be accounted for only by the experimental deviation of 2-fold. We, therefore, correlated the level of *TEL-AML1* with the WBC counts of the patients at presentation as a measure of leukemia burden. As shown in Fig. 6*B*, no correlation was apparent even when only the major group, *i.e.*, patients with $<30 \times 10^9$/liter, was analyzed. However, three groups of patients could be discerned: (*a*) majority clustered within a level of expression between 100 and 600 (34 of 47, 72%); (*b*) small proportion expressed lower values, <100 (6 of 47, 13%); and (*c*) small group expressed higher levels, >600 (7 of 47, 15%). Although the number of patients in each group is small, we correlated these three categories with clinical data. We did not find statistically significant association with gender, age, hemoglobin, platelet count, or presence of hepatomegaly or splenomegaly (Pearson χ^2 test). Similarly, overall survival was not significantly different between the groups (83.33, 91.83, and 100% for the low, moderate, and high expressing categories, respectively).

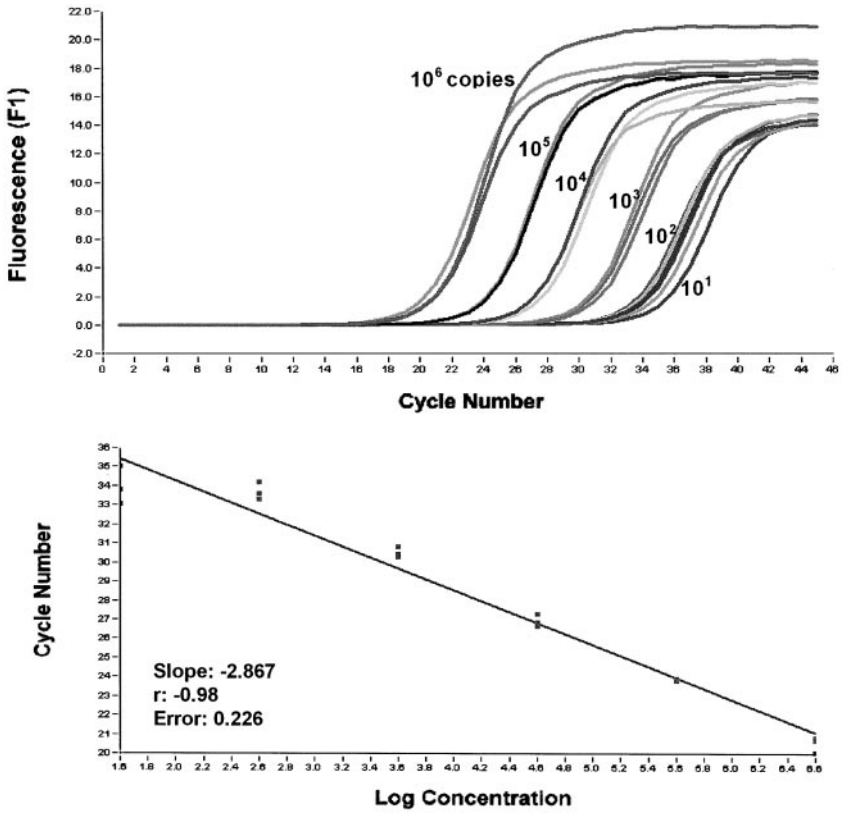

Fig. 5 Quantification of *TEL-AML1* levels by real-time RT-PCR. The *top graph* shows the kinetics of the reaction for six serial dilutions of plasmid DNA containing the *TEL-AML1* amplicon run in triplicates. The fluorescence is measured in every PCR cycle, and it is proportional to the accumulation of PCR product. Amounts over five logs can be unequivocally quantified. Concentration was measured as number of transcripts. The *bottom panel* shows the standard curve in which the Cp (Y axis) is a function of the logarithm of the concentration. The Cp values are inversely proportional to the log of the initial template amounts. The parameters from the Lightcyler software are shown.

DISCUSSION

The presence of translocations in ALL not only influences the biology of the leukemic clone but also predicts clinical outcome. Therefore, we developed a simple real-time RT-PCR multiplex assay to detect the four most common rearrangements in childhood precursor B ALL. There are recent reports based on real-time RT-PCR to detect some of these translocations (15–18), but all these assays detect a single translocation and use dual color fluorochromes in the Taqman system. Therefore, the major advantages of this newly developed test are that it is a multiplex reaction and uses only Sybr green I. This monochrome assay allows reliable identification of the amplicons based on their characteristic melting peak.

We tested several primer pairs to arrive at the sets that reproducibly amplified a segment with a characteristic peak and very little variability (0.3–0.5°C), allowing proper identification (Table 1) both singly and in a multiplex reaction. Three of the translocations were easily combined (Figs. 1 and 2*A*), but amplification of m*BCR-ABL* was always compromised under these conditions and was run in a tandem reaction (Fig. 2*B*).

The primers were designed to amplify the two known variants of *TEL-AML1*, m*BCR-ABL*, and *E2A-PBX*1. MLL-*AF4* has been reported with at least 10 different rearrangements, and the primers used here amplify 6 of them representing >85% of all t(4;11) (20). We have also tested another *AF4* primer (in exon 8), which will expand the amplifiable rearrangements to >95%. Interestingly, this new amplicon has a very similar melting peak (83.9 ± 0.5) to the previous MLL-*AF4* PCR product and can be easily interchanged. We attempted to use another set of primers that will amplify all known 4;11 variants (exon 8 of each gene), but this amplification was compromised in the multiplex reaction.

To have an internal control in the multiplex reaction, seven housekeeping genes were tested and only one, *HPRT*, yielded an appropriate melting peak not overlapping with the ones from fusion transcripts in the multiplex reaction (Fig. 2*A*). This was important when testing samples from patients for the potential translation to the clinical setting. Every sample yields a specific product from the housekeeping gene or from the fusion transcript if a translocation is present ensuring PCR ability of every cDNA and reconfirming the high fidelity of the system.

In fact, this assay was validated with 89 ALL pediatric cases from two non-Western countries. Although the numbers of patients were small and the study was not meant to be a comprehensive epidemiological study, it appears that in comparison to the West, *E2A-PBX*1 and *BCR-ABL* ALLs were underrepresented in Saudi Arabia (0 of 40) but not in India (4 of 52) (Table 3). One possible explanation is that although our cohort of Indian patients had a median age of 7 years, the median age for the Arab patients studied was 3 years. The t(1;19) as well as the t(9;22) most frequently occur in older patients (11, 12). On the other hand, *TEL-AML1* occurred at a significantly low frequency in ALL from India (4%) but not in Saudi Arabia (20%), reconfirming previous data (19). Additional studies of larger number of patients are warranted to confirm these results.

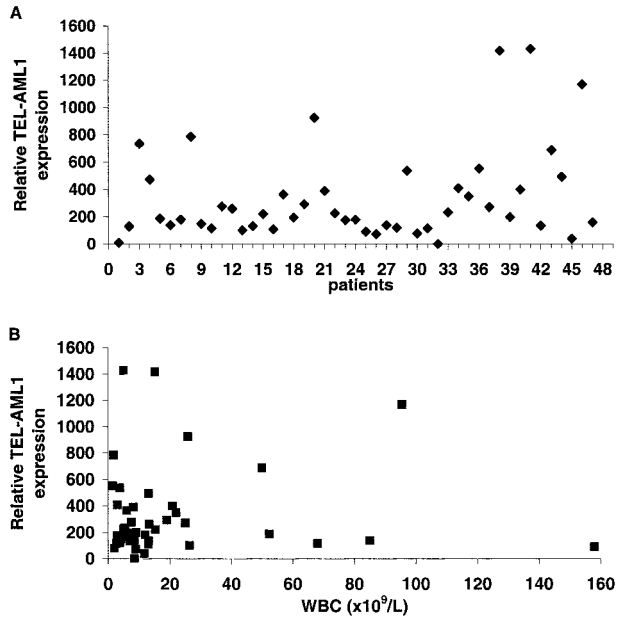

Fig. 6 Quantification of *TEL-AML1* levels in patients with t(12;21) calculated as the relative number of transcripts/cell. The variability of expression in individual patients is shown in *A*. The *graph* in *B* shows that there is no correlation between the level of expression of *TEL-AML1* and WBC counts (surrogate marker of leukemia burden).

It is clear that this monochrome real-time multiplex RT-PCR assay has high specificity and allows fast, high throughput screening of therapy-relevant rearrangements. Previous reports have demonstrated the advantages of multiplexing PCRs for screening translocations in ALL (22–27), and to the best of our knowledge, this is the first report of a real-time multiplex assay for translocations.

Apart from this qualitative analysis, we also demonstrate that the monochrome real-time RT-PCR can be used in quantification analyses. Real-time quantification is based on the amount of fluorescence produced in a reaction, which is proportional to the starting target amount during the early phases of amplification. Hence, the cycle in which the signal reached the threshold (Cp) is used. Because the initial qualitative analysis suggested that *TEL-AML1* levels varied enormously between patients, we developed a standard curve for this fusion transcript and for *GAPDH* as a control (Figs. 4 and 5). Under our conditions, one positive cell could be detected in a background of 100,000 normal cells. This sensitivity is similar to that reported with the Taqman assay (28–30), indicating the potential use of this less expensive test for studies of minimal residual disease.

The translation of the assay to a clinical setting must also take into account that when samples from patients are studied, several factors can affect quantification. These include primarily the quality of RNA because of variations in transport and processing times, the efficiency of the enzymatic reactions (reverse transcription and amplification) and handling errors. Therefore, we calculated in our conditions an inter experiment and intraexperiment variability of $<$2-fold.

TEL-AML1 was quantified in 47 positive samples, and we observed variable levels of the fusion transcript beyond the experimental deviation (Fig. 6*A*). This was somewhat surprising because this translocation retains the *TEL* promoter, and therefore, it would be expected to be consistently regulated in all *TEL-AML1* leukemias. Our observation then could indicate heterogeneity of other factors influencing the transcript levels. Three groups of patients appear to exist, a cluster (72%) with arbitrary expression of 100–600, low expressors (13%) and high expressors (15%). No statistical association with WBC (Fig. 6*B*), gender, age, hemoglobin, platelets, organomegaly, or overall survival was detected. Because this variation appears to be an intrinsic factor, it may influence the biology of this ALL. Additional correlations may elucidate the clinical significance of this observation.

Previous reports have quantified *TEL-AML1* levels in a few cases to study minimal residual disease and, consequently, the kinetics of disappearance of leukemic transcripts during therapy (28–30). Although different *TEL-AML1* levels at presentation were not specifically highlighted, Pallisgaard *et al.* (17) reported up to 14-fold variation in 12 patients. From the data of Drunat *et al.* (29), 6.5-fold differences can be deduced and this variation appeared to be independent of the percentage of cycling cells.

In conclusion, our real time quantitative-RT-PCR using Sybr green I represents a robust, reliable, sensitive, and cost effective technique for quantitative PCR analyses. The turnover time is short, and the capacity for sample throughput is high. Furthermore, this strategy allows correlation of outcome not only with the type of gene rearrangements but also has the simultaneous potential of quantifying the expression of chimeric genes in ALL at presentation, making the follow up of minimal residual disease feasible.

REFERENCES

1. Pui, C. H. Recent advances in the biology and treatment of childhood acute lymphoblastic leukemia. Curr. Opin. Hematol., *5:* 292–301, 1998.

2. Rubnitz, J. E., and Look A. T. Molecular genetics of childhood leukemia. J. Pediatr. Hematol. Oncol., *20:* 1–11, 1998.

3. Pui, C. H. Acute lymphoblastic leukemia in children. Curr. Opin. Oncol., *12:* 3–12, 2000.

4. Look, A. Fusion genes and their hybrid proteins in acute lymphoblastic leukemia. Educational book, 30th annual meeting of the American Society of Clinical Oncology. Proc. Am. Soc. Clin. Oncol. Annu. Meet., 131–138, 1994.

5. Maloney, K., McGavran, L., Murphy, J., Odom, L., Stork, L., Wei, Q., and Hunger, S. *TEL-AML1* fusion identifies a subset of children with standard risk acute lymphoblastic leukemia who have an excellent prognosis when treated with therapy that includes a single delayed intensification. Leukemia (Baltimore), *13:* 1708–1712, 1999.

6. Behm, F. G., Raimondi, S. C., Frestedt, J. L., Liu, Q., Crist, W. M., Downing, J. R., Rivera, G. K., Kersey, J. H., and Pui, C. H. Rearrangement of the MLL gene confers a poor prognosis in childhood acute lymphoblastic leukemia, regardless of presenting age. Blood, *87:* 2870–2877, 1996.

7. Pui, C. H., Raimondi, S. C., Hancock, M. L., Rivera, G. K., Ribeiro, R. C., Mahmoud, H. H., Sandlund, J. T., Crist, W. M., and Behm, F. G. Immunologic, cytogenetic and clinical characterization of childhood acute lymphoblastic leukemia with the t(1;19)(q23:p13) or its derivative. J. Clin. Oncol., *12:* 2601–2606, 1994.

8. Crist, W., Carroll, A., Shuster, J., Jackson, J., Head, D., Borowitz, M., Behm, F., Link, M., Steuber, P., Ragab, A., *et al.* Philadelphia chromosome positive childhood acute lymphoblastic leukemia: clinical

and cytogenetic characteristics and treatment outcome. A Pediatric Oncology Group Study. Blood, *76:* 489–494, 1990.

9. Bennett, J. M. World Health Organization classification of the acute leukemias and myelodysplastic syndrome. Int. J. Hematol., *72:* 131–132, 2000.

10. Uckun, F. M., Pallisgaard, N., Hokland, P., Navara, C., Narla, R., Gaynon, P. S., Sather, H., and Heerema, N. Expression of *TEL-AML1* fusion transcripts and response to induction therapy in standard risk acute lymphoblastic leukemia. Leuk. Lymphoma, *42:* 41–56, 2001.

11. Uckum, F. M., Sensel, M. G., Sather, H. N., Gaynon, P. S., Arthur, D. C., Lange, B. J., Steinherz, P. G., Kraft, P., Hutchinson, R., Nachman, J. B., Reaman, G. H., and Heerema, N. A. Clinical significance of translocation t(1;19) in childhood acute lymphoblastic leukemia in the context of contemporary therapies: a report from the Children's Cancer Group. J. Clin. Oncol., *16:* 527–535, 1998.

12. Schlieben, S., Borkhardt, A., Reinisch, I., Ritterbach, J., Janssen, J. W. G., Ratei, R., Schrappe, M., Repp, R., Zimmermann, M., Kabisch, H., Janka-Schaub, G., Bartram, C. R., Ludwig, W-D., Riehm, H., Lampert, F., and Harbott, J. Incidence and clinical outcome of children with *BCR/ABL*-positive acute lymphoblastic leukemia (ALL). A prospective RT-PCR study based on 673 patients enrolled in the German pediatric multicenter therapy trials ALL-BFM-90 and CoALL-05-92. Leukemia (Baltimore), *10:* 957–963, 1996.

13. Uckun, F. M., Herman-Hatten, K., Crotty, M. L., Sensel, M. G., Sather, H. N., Tuel-Ahlgren, L., Sarquis, M. B., Bostrom, B., Nachman, J. B., Steinherz, P. G., Gaynon, P. S., and Heerema, N. Clinical significance of MLL-*AF4* fusion transcript expression in the absence of a cytogenetically detectable t(4;11)(q21;q23) chromosomal translocation. Blood, *92:* 810–821, 1998.

14. Wittwer, C. T., Herrmann, M. G., Moss, A. A., and Rasmussen, R. P. Continuous fluorescence monitoring of rapid cycle DNA amplification. Biotechniques, *22:* 130–138, 1997.

15. Emig, M., Saubele, S., Wilton, H., Welber, A., Retler, A., Willer, A., Berger, U., Hehlmann, R., Cross, N. C. P., and Hochaus, A. Accurate and rapid analysis of residual disease in patients with CML using specific fluorescent hybridization probes for real-time quantitative RT-PCR. Leukemia (Baltimore), *13:* 1825–1832, 1999.

16. Curry, J. D., Glasser, M. C., and Smith, M. T. Real-time reverse transcription polymerase chain reaction detection and quantification of t(1;19) (*E2A-PBX*1) fusion genes associated with leukemia. Br. J. Haematol., *115:* 826–830, 2001.

17. Pallisgaard, N., Clausen, N., Schroder, H., and Hokland, P. Rapid and sensitive minimal residual disease detection in acute leukemia by quantitative real-time RT-PCR exemplified by t(12;21) *TEL-AML1* fusion transcript. Genes Chromosomes Cancer, *26:* 355–365, 1999.

18. Ballerini, P., Landman Parker, J., Laurendeau, I., Olivi, M., Vidaud, M., Adam, M., Leverger, G., Gerota, I., Cayre, Y. E., and Bieche, I. Quantitative analysis of *TEL*/*AML1* fusion transcripts by real-time RT-PCR assay in childhood acute lymphoblastic leukemia. Leukemia (Baltimore), *14:* 1526–1531, 2000.

19. Inamdar, N., Kumar, S. A., Banavali, S. D., Advani, S., Magrath, I., and Bhatia, K. Comparative incidence of the rearrangements of *TEL*/*AML1* and *ALL1* genes in pediatric precursor B acute lymphoblastic leukemias in India. Int. J. Oncol., *3:* 1319–1322, 1998.

20. van Dongen, J. J. M., Macintyre, E. A., Gabet, J. A., Delabesse, E., Rossi, V., Saglio, G., Gottardi, E., Rambaldi, A., Dotti, G., Griesinger, F., Parreira, A., Gameiro, P., Gozalez Diaz, M., Malec, M., Langerak, A. W., San Miguel, J. F., and Biondi, A. Standardized RT-PCR analysis of fusion gene transcripts from chromosome aberrations in acute leukemia for detection of minimal residual disease. Report of the BIOMED-1 concerted action. Leukemia (Baltimore), *13:* 1901–1928, 1999.

21. Numata, S., Kato, K., and Horibe, K. New *E2A-PBX*1 fusion transcript in a patient with t(1;19)(q23;p13) acute lymphoblastic leukemia. Leukemia (Baltimore), *7:* 1441–1444, 1993.

22. Izraeli, S., and Lion, T. Multiprimer PCR for screening of genetic abnormalities in acute lymphoblastic leukemia. Br. J. Haematol., *79:* 645–647, 1991.

23. Scurto, P., Rocha, M., Kane, J., Williams, W., Haney, D., Conn, W., Shurtleff, S., and Downing, J. A multiplex RT-PCR assay for the detection of chimeric transcripts encoded by the risk-stratifying translocations of pediatric ALL. Leukemia (Baltimore), *12:* 1994–2005, 1998.

24. Pallisgaard, N., Hokland, P., Riishoj, D. C., Pedersen, B., and Jorgensen, P. Multiplex reverse transcription-polymerase chain reaction for simultaneous screening of 29 translocations and chromosomal aberrations in acute leukemia. Blood, *92:* 574–588, 1998.

25. Viehmann, S., Borkhardt, A., Lampert, F., and Harbott, J. Multiplex PCR: a rapid screening method for detection of gene rearrangements in childhood acute lymphoblastic leukemia. Ann. Hematol., *78:* 157–162, 1999.

26. Cerveira, N., Ferreira, S., Doria, S., Veiga, I., Ferreira, F., Mariz, J. M., Marques, M., and Castedo, S. Detection of prognostic significan translocations in childhood acute lymphoblastic leukemia by one-step multiplex reverse transcription polymerase chain reaction. Br. J. Haematol., *109:* 638–640, 2000.

27. Marin, C., Martinez-Delgado, B., Melendez, B., Larrayoz, M. J., Martinez-Ramirez, A., Robledo, M., Cigudosa, J. C., Calasanz, M. J., and Benitez, J. Multiplex polymerase chain reaction assay for the detection of prognostically significant translocations in acute lymphoblastic leukemia. Haematologica, *86:* 1254–1260, 2001.

28. Seeger, K., Kreuzer, K-A., Lass, U., Taube, T., Buchwald, D., Eckert, C., Korner, G., Schmidt, C-A., and Henze, G. Molecular quantification of response to therapy and remission status in *TEL-AML1*-positive childhood ALL by real-time reverse transcription polymerase chain reaction. Cancer Res., *61:* 2517–2522, 2001.

29. Drunat, S., Olivi, M., Brunie, G., Grandchamp, B., Vilmer, E., Bieche, I., and Cave, H. Quantification of *TEL-AML1* transcript for minimal residual disease assessment in childhood acute lymphoblastic leukaemia. Br. J. Haematol., *114:* 281–289, 2001.

30. de Haas, V., Breunis, W. B., Dee, R., Verhagen, O. J., Kroes, W., van Wering, E. R., van Dongen, J. J., van der Berg, H., and van der Schoot, C. E. The *TEL-AML1* real-time quantitative polymerase chain reaction (PCR) might replace the antigen receptor-based genomic PCR in clinical minimal residual disease studies in children with acute alymphoblastic leukemia. Br. J. Haematol., *116:* 87–93, 2002.

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Clin Cancer Res 2002;8:3832-3840.