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Quantitative Detection of PML-RARα Fusion Transcript by Real-Time PCR with A Single Primer Pair

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Quantitative detection of minimal residual disease has prognostic value for some leukemias. Acute promyelocytic leukemia (APL) is characterized by the specific PML-RARα fusion gene from t(15;17). Added to three PML-RARα isoforms, alternative spliced forms of PML exons give rise to multiple isoforms even within a single patient. To date, multiple primer pairs for the detection of the various PML-RARα transcripts have been designed, potentially generating some nonspecific amplification products. Here, we established a real-time quantitative PCR (RQ-PCR) strategy with a single primer pair using LightCycler (sp-RQ-PCR), which could simultaneously detect three isoforms with equal specificity and sensitivity as well as alternative spliced forms. Results obtained with sp-RQ-PCR for 39 samples from 15 APL patients and 31 non-APL samples were compared with those with TaqManTM assay with three primer pairs. In two of the APL samples, PML-RARα was detected in the TM, but not in the sp-RQ-PCR or nested PCR. Furthermore, the sp-RQ-PCR showed no positive results for the 31 non-APL samples, whereas the TM identified 13% (4/31) as positive. Electrophoresis detected some artifacts in the TM, which do not correspond to PML-RARα. We conclude that our sp-RQ-PCR is specific enough to identify various forms of PML-RARα and yields no false-positive results. J. Clin. Lab. Anal. 23:223–230, 2009.

Key words: real-time quantitative PCR; PML-RARα isoform; single primer pair; alternative splicing; lightcycler

INTRODUCTION

Acute promyelocytic leukemia (APL) is characterized by the specific PML-RARα fusion gene resulting from translocation t(15;17)(q22;q12). The breakpoint on chromosome 17 invariably is located within intron 2 of the RARα gene (1,2). The fusion transcript variation is the result of the presence of heterogeneous breakpoint cluster regions (*bcr*) within the PML gene and of alternative splicings of the *PML* sequence following transcription (3–5). As three regions of the PML locus are involved in the t(15;17) translocation breakpoints: intron 6 (*bcr1*; 55% of cases), exon 6 (*bcr2*; 5%), and intron 3 (*bcr3*; 40%), there are three possible PML-RARα isoforms, known as long (L, or *bcr1*), variant (V, or *bcr2*), and short (S, or *bcr3*). The *bcr*2 breakpoint occurs at inconsistent sites within exon 6 of the PML gene, resulting in the fusion of variable segments of the PML exon 6 with exon 3 of the RARα gene (6–8). Heterogeneity of the chromosome 15 breakpoint accounts for differences in the architecture of the PML-RARα mRNAs isolated from different APL patients, whereas alternative spliced forms of PML exons give rise to multiple isoforms of the PML-RARα mRNAs even within a single patient (3,9).

Although almost all patients achieve complete remission within 1–3 months, nearly 30% of these patients eventually relapse (10,11). Some studies have reported clinical relevance of the specific type of PML-RARα

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fusion transcript expressed in an individual, which suggests that APL-positive cases carrying the short-form fusion transcript tend to have shorter periods of clinical remission than do patients with the long form (12–14). In addition, cases carrying the variant form may have a diminished response to treatment with all-*trans* retinoic acid, depending on where the break occurs within PML exon 6 (15).

Detection of minimal residual disease (MRD) is of prognostic value for several types of leukemias, which our previous study proved for AML1/MTG8 (16). Quantitative MRD data can be obtained with real-time quantitative PCR (RQ-PCR) analysis of fusion transcript levels before, during, and after therapy (17,18). Although PML-RARα was one of the first targets used for MRD detection in clinical studies, conventional nested reverse transcriptase-polymerase chain reaction (RT-PCR) (19) is still being used, even in recently reported studies (12,20). Studies using quantitative assessment of PML-RARα are rare and need to be increased. To date, multiple primers that allow the detection of the various PML-RARα fusion transcripts have been designed for the RQ-PCR, but mostly for the TaqManTM system, which may have the advantage that the RQ-PCR can detect the various types of PML-RARα at one time. However, some studies have reported that such RQ-PCR assays for PML-RARα show false-positive results, which can be attributed to some nonspecific amplification products potentially generated by multiple primer sets (21,22). Here, we established a RQ-PCR strategy with a single primer pair (sp-RQ-PCR) using LightCycler (LC) (Roche Diagnostics, Basel, Switzerland) technology for a procedure that is a modification of the one described by Schnittger et al., which uses two forward primers separately (tps-RQ-PCR) (23). The aim of our study was to determine, in a comparative analysis with the TM assay, the specificity and sensitivity of this strategy for the detection of *bcr1*, *bcr2* and *bcr3* isoforms as well as of their alternative spliced forms. Moreover, we demonstrate that there are no false-positive results detected by the sp-RQ-PCR, as there are with the TM assay. Our data suggest that our sp-RQ-PCR can be used to quantify changes in PML-RARα expression during treatment and be helpful for the prediction of the possibility of relapse (24,25).

MATERIALS AND METHODS

Patients and Samples

The NB4 cell line was used as a positive control for the *bcr1* long form. The positive controls for the *bcr3* and *bcr2* forms were derived from positive patient samples carrying the t(15;17) short and variant forms. The K562 cell line was used as a negative control. Between 2003 and 2008, 39 bone marrow or peripheral blood samples were obtained from 15 APL patients and 31 from non-APL leukemia patients. The 15 cases were confirmed as carrying t(15;17) by karyotyping or FISH, and presence of the PML-RARα fusion transcript was confirmed with conventional RT-PCR. De novo samples from the 15 patients showed high levels of blast cells, and high-quality RNAs were selected for the RQ-PCR assay and nested PCR. Informed consent for all samples was provided according to the Declaration of Helsinki.

RT-PCR Qualitative Assays

Total RNA was isolated with the RNeasy Mini kit (Qiagen, Hilden, Germany) and cDNA was constructed from 1 μg of total RNA with the First Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche Diagnostics, Tokyo, Japan) according to the manufacturer's instructions. All samples were subjected to qualitative RT-PCR. cDNA was amplified by using two-step nested PCR in a 2 mmol/L $MgCl_2$, 200 mmol/L dNTP, 10 × PCR Gold Buffer containing 15 mmol/L Tris-HCL (pH 8.0) and 50 mmol/L KCl, and 1.25 U of AmpliTaq Gold (Applied Biosystems, Foster City, CA), using 0.2 μmol/L of each of the primers P1, 5′-ATGCTGT-GCTGCAGCGCAT-3′ and R7, 5′-CCATAGTGGTA-GCCTGAGGAC-3′. After 10 min of incubation at 95°C, PCR was performed at 35 cycles at 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec. Five microliter of 50 μL each from the first-step PCR product were amplified with the nested primers P2, 5′-CAGAGGATGAAGTGCTA-CG-3′ and R9, 5′-GTCCTGACAGACAAAGCAAG-3′ under the same conditions as those for the first-step PCR, followed by electrophoresis on a 2% agarose gel. Classification of the specific PML-RARα isoforms, *bcr1* (757 bp), *bcr2* (496 bp), and *bcr3* (284 bp), was evaluated for 15 APL patients.

Standard Preparation

Two fusion transcripts including *bcr1* were amplified in RT-PCR from NB4 and cloned into a plasmid vector (TOPO TA cloning kit, Invitrogen, Groningen, The Netherlands). *Bcr3* and *bcr2* were cloned from patients carrying the corresponding breakpoint. The cloned products were then sequenced by means of Big Dye Terminator Cycle Sequencing for an ABI Prism 3130 × l sequence detection system (Applied Biosystems). Finally, the products were purified (PCR Purification Kit; Qiagen) and measured in a NanoDrop (NanoDrop Technologies, Wilmington, DE), after which the molecule concentrations were calculated. Each plasmid cDNA of PML-RARα was then serially diluted over a range from 10^{-8} to 10^{-17} mol/L with TE buffer (RNase, DNase protease-free; Nakalai Tesque, Kyoto, Japan).

Five plasmid cDNAs of PML-RARα were prepared for TM assay in a similar manner.

RQ-PCR (Real Time) Assays

Total RNA was extracted and reversely transcribed as described above. All primers and hydrolysis and hybridization probes used for this study are listed in Table 1 For the TM assay, the RQ-PCR with three primer pairs was performed using the ABI Prism 7700 DNA Sequence Detection System (Applied Biosystems) according to the Europe Against Cancer Group (EAC) protocol (22). The original LC assay described by Schnittger et al. used one forward primer either in PML exon 6 for *bcr1* or in exon 3 for *bcr3*, a reverse primer, and hybridization probes in *RARα* exon 3 (tps-RQ-PCR) (23). However, the sp-RQ-PCR was carried out with one forward primer *bcr3* in exon 3 and PCR conditions were modified, using a 20 μL reaction volume for each 0.25 μmol/L of the *bcr3* forward and reverse primers, 0.25 μmol/L Hyb-Probes, 3 mmol/L $MgCl_2$, 2 μL LC-FastStart DNA Master Hybridization Probes (Roche Diagnostics, Indianapolis, IN), and 2 μL each of the cDNAs. Samples were incubated at 32°C for 5 min, at 94°C for 10 min, followed by 45 cycles, at 95°C for 10 sec, 60°C for 10 sec, and 72°C for 35 sec. To assess the quality and quantity of the isolated RNA as well as the efficiency of cDNA synthesis, each sample was normalized against the corresponding expression of GAPDH. PML-RARα negative cells and blank controls without a template were tested as negative controls. Plasmid cDNAs obtained from NB4 were also used as positive controls.

LC data were analyzed using LightCycler 3.0 software. Ten-fold dilution series of five different plasmid concentrations were used for standard curves. As the slope of the standard curve is an indirect measure of PCR efficiency, values between 3.7 and 3.0 were considered to be indicators of an acceptable PCR system. Moreover, for a more precise quantification, standard curves were accepted with a correlation coefficient of at least 0.95 ($r > 0.95$). In all RQ-PCR assays, cycle threshold (C_T) values more than 40 were not considered PCR positive.

RESULTS

Standard Curves

This RQ-PCR assay with a single primer pair (sp-RQ-PCR) was evaluated for sensitivity, specificity, reproducibility, and compared with those of the TM assay with three primer pairs. Figure 1 shows a schematic diagram of the fusion gene transcripts amplified from NB4 (P1 and P2) and from the patients carrying the corresponding breakpoints (P3 and P4). Figure 2 shows LC fluorescence detection during the sp-RQ-PCR of the dilution series of plasmid cDNAs cloned from the four PML-RARα fusion transcripts (P1–P4). These amplification plots demonstrated a high degree of similarity, as did those of the TM assay (data not shown). Figure 3 illustrates standard curves obtained by plotting the initial number of target molecules in the dilution series of plasmid cDNAs against their corresponding cycle threshold (C_T). This procedure is based on the background fluorescence intensity detected during the first 3 to 15 PCR cycles, after which a threshold is determined. C_T is defined as the PCR cycle at which the fluorescence exceeds the threshold for the first time. The C_T values are directly proportional to the amount of the target sequence present in the samples. For all the sp-RQ-PCR in Figure 2, the standard curves yielded a very

TABLE 1. Sequences and Positions of the PML-RARα Primers and Probes

	Primer and probes 5′–3′	GenBank accession no.	Position
TM assay[a]			
ENF903 PML	TCTTCCTGCCCAACAGCAA	M73778	1690–1708
ENF906 PML	ACCTGGATGGACCGCCTAG	M73778	1642–1660
ENF905 PML	CCGATGGCTTCGACGAGTT	M73778	1198–1216
ENF942 RARA	FAM-AGTGCCCAGCCCTCCCTCGC-TAMRA	X06538	439–458
ENF962 RARA	GCTTGTAGATGCGGGGTAGAG	X06538	485–465
sp-RQ-PCR[b]			
Primer bcr3	AGCTCTTGCATCACCCAGGGGA	M73778	1239–1260
Primer reverse	CTCACAGGCGCTGACCCCATAGT	X06538	522–544
Sensor	TGAAGAGATAGTGCCCAGCCCTCCCTC-FL	S50916	66–92
Anchor	LCRed640-CCACCCCCTCTACCCCGCATCTACA	S50916	91–118
Primer bcr1[c]	GTCTTCCTGCCCAACAGCAACC	M73778	1689–1710

TM, TaqMan assay; sp-RQ-PCR, real-time quantitative PCR with a single primer pair; FAM, 6-carboxy fluorescein; TAMRA, 6-carboxytetramethyl rhodamine; LCRed640, LightCycler Red 640; FL, fluorescein.

[a]The primers and probes for TM assay using ABI prism 7700 are described by Gabert et al.

[b]Those for sp-RQ-PCR using LightCycler for a procedure that is a modification of the one described by Schnittger et al.

[c]Schnittger et al. uses two forward primers including bcr1 and bcr3.

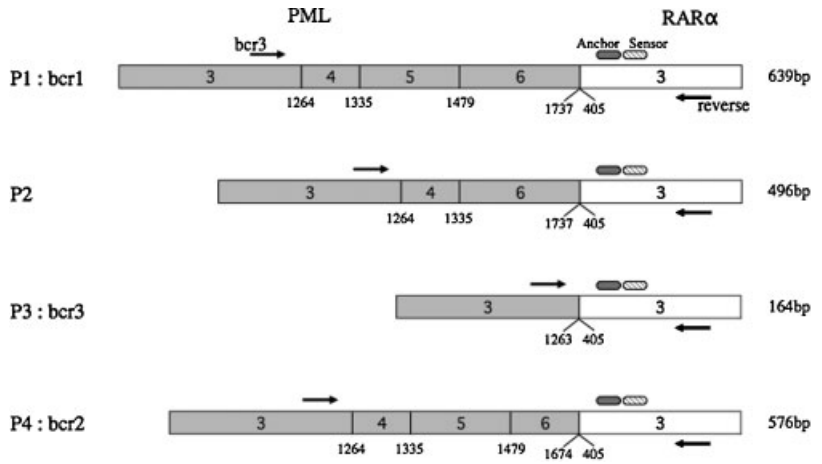

Fig. 1. Schematic diagram of the PML-RARα fusion gene transcripts covered by primers and probes of RQ-PCR. For sp-RQ-PCR, the forward primer bcr3 is used. P1 and P2 indicate amplification products from NB4, and P3 and P4 from patients carrying the respective breakpoints. The arrows on the right represent forward primers, and the arrows on the left reverse primers. The box-like shapes represent probes. Sequences of primers and probes are listed in the Table 1.

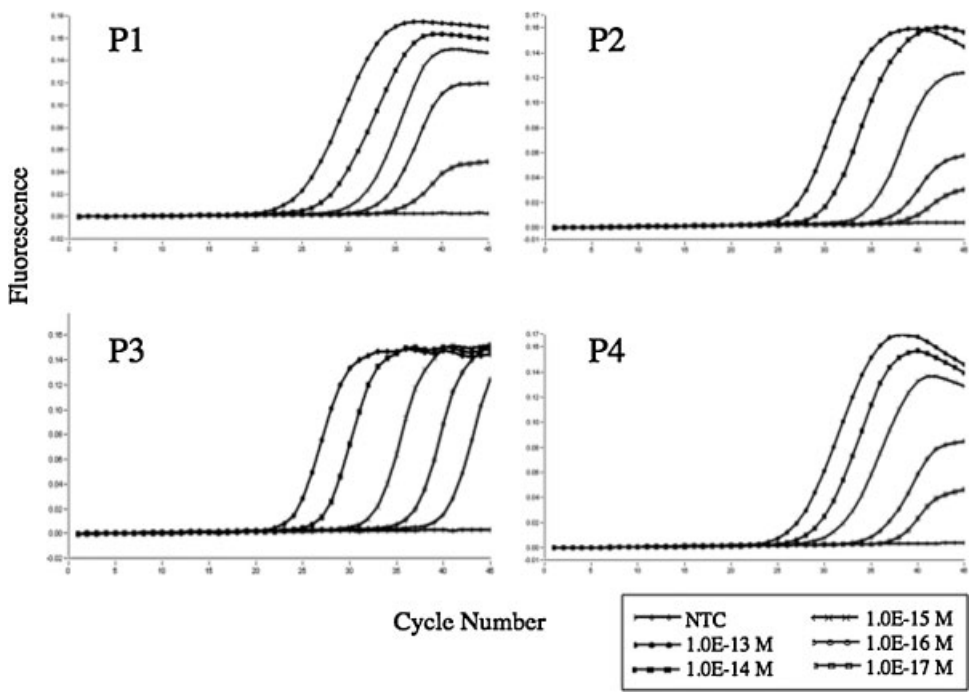

Fig. 2. LightCycler amplification plots of four PML-RARα plasmid cDNAs using sp-RQ-PCR. The figures show amplification plots from ten-fold serial dilutions of the cDNAs shown in Figure 1. The relative change in fluorescence during cycling is displayed on the *Y*-axis. Inset: absolute numbers of starting templates (1.0E–16 mol/L, 10^{-16} mol/L; 1.0E–17, 10^{-17}, and so on). NTC: nontemplate controls.

consistent C_T for each of the points with a correlation coefficient of 0.99, indicating the linearity ranged over five orders of magnitude from 10^{-13} to 10^{-17} mol/L. The medium slope value of the standard curve was −3.60, indicating that all four assays featured similar kinetics and near optimum PCR amplification (−3.3) (Fig. 3). Similar results were obtained from the TM assay (data not shown), where the TM assay was performed by using the five dilution series of the plasmid cDNAs specially produced for it. The TM assay was also capable of maintaining reproducible linearity over the entire dilution series with slopes of approximately −3.45, and correlation coefficients of more than 0.99 (data not shown).

Reliability of the Assay

The inter-assay and intra-assay coefficient of variation (CV) was calculated for every reaction to evaluate the

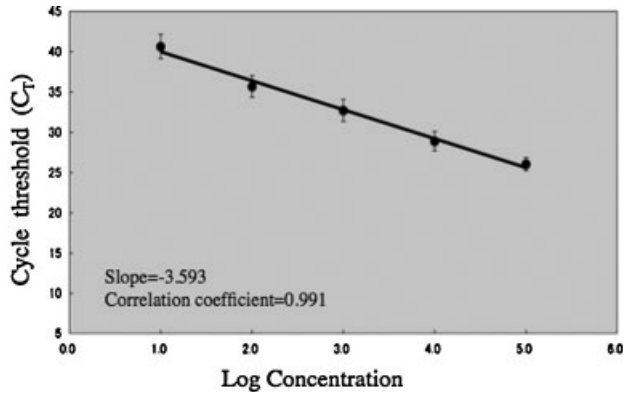

Fig. 3. A standard curve from the LC assay using a single primer pair (sp-RQ-PCR). The figure shows the mean threshold cycle (C_T) for repetitive analysis of four PML-RARα fusion standards (P1–P4). The slope of the standard curve is close to the theoretical slope of −3.3.

reproducibility of the assays. The inter-assay reproducibility of the long-form reaction, determined by comparing C_T values from 10^{-11} and 10^{-16} mol/L of P1 plasmid for eight different runs, showed CVs of 2.0 and 2.3%, respectively, whereas that of the short-form reaction (P3) showed CVs of 3.6 and 2.9%, respectively. The intra-assay CVs for the long- and short-form reactions, calculated from the C_T values of 10 replicates of P1 and P3 plasmids from a single run, were 1.8 and 3.3% and 1.4 and 1.9%, respectively.

Sensitivity

When the sensitivity was studied in serial ten-fold dilutions of 1 μg of total RNA of NB4 cells diluted in K562 cells, the sp-RQ-PCR achieved a sensitivity of 10^{-4}, corresponding to one PML-RARα positive cell among 10^4 negative cells. Ten-fold dilutions of cDNA obtained from RT of NB4 cells diluted in TE buffer were also tested. A dilution of 5×10^{-4} was detectable when 1 μg of total RNA was transcribed. Although these results were consistent with those for the TM assay, the nested PCR detected a dilution of 10^{-5} in the same dilution samples, indicating that the sp-RQ-PCR was less sensitive than the nested RT-PCR.

Quantitative PML-RARα and Patient Profiles

PML-RARα expression was studied in 39 samples from 15 APL patients, nine with *bcr1*, five with *bcr3*, and one with *bcr2*, and 31 samples from non-APL leukemia patients. We compared the absolute amounts of template molecules obtained from the sp-RQ-PCR and TM assay (Table 2). The same samples were also tested with nested PCR. PML-RARα transcript generated by two assays correlated well for 15 positive samples, with a correlation coefficient of 0.922 (data not shown). When

TABLE 2. Quantification Results for PML/RARα Transcripts Measured With sp-RT-PCR and TaqMan Assays

		PML-RARα/GAPDH∗10^6		
Patient #	Isoform	sp-RQ-PCR	TM	Nested PCR (positive/test)
1–1	bcr1	11100	1480	2/2
1–2		66.8	27.1	2/2
1–3		20.3	22.6	1/4
1–4		ND	27.6	0/2
1–5		ND	ND	0/2
1–6		ND	ND	0/2
1–7		ND	ND	0/2
1–8		ND	5.61	0/2
1–9		ND	ND	0/2
2–1	bcr1	1210	1640	2/2
2–2		ND	ND	0/2
2–3		ND	ND	0/2
2–4		ND	ND	0/2
2–5		ND	ND	0/2
2–6		ND	ND	0/2
2–7		ND	ND	0/2
2–8		ND	ND	0/2
2–9		ND	ND	0/2
3–1	bcr3	86.9	5.61	2/2
3–2		76.5	15.5	2/2
3–3		4010	1540	2/2
3–4		37.7	16.9	2/2
3–5		ND	ND	0/2
4–1	bcr1	33.9	52.5	2/2
4–2		ND	ND	0/2
4–3		ND	ND	0/2
5–1	bcr1	18900	10300	2/2
5–2		12500	9950	2/2
6–1	bcr2	128	390	2/2
6–2		11.4	106	1/6
7	bcr3	3230	2650	2/2
8	bcr3	416000	275000	2/2
9	bcr3	ND	ND	4/6
10	bcr3	ND	ND	0/2
11	bcr1	ND	ND	0/2
12	bcr1	ND	ND	0/2
13	bcr1	ND	ND	0/2
14	bcr1	ND	ND	0/2
15	bcr1	ND	ND	0/2

sp-RQ-PCR, real-time quantitative PCR with a single primer pair using LightCycler; TM, TaqMan assay using ABI Prism 7700; ND, not detect.

C_T values more than 40 were not defined as PCR positive, in two cases (♯1–4, 1–8), PML-RARα transcript was detected with the TM assay, but not with the sp-RQ-PCR and nested PCR. These amplification products detected by the sp-RQ-PCR and TM assay were then checked by electrophoresis on a 2% agarose gel and with ethidium bromide staining, which showed the TM assay detected a nonspecific band, as confirmed by sequencing analysis (Fig. 4, ♯1–4). As seen in Figure 4, ♯1–1, both assays detected alternative spliced bands added to the long-form band, whereas the TM assay

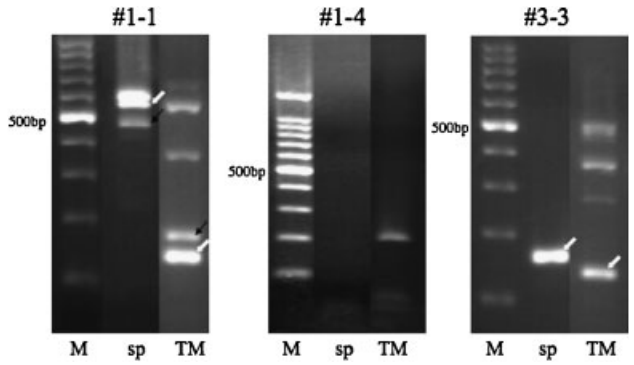

Fig. 4. Electrophoresis of amplification products after sp-RQ-PCR and TM assay. Amplification products of the samples (♯1–1, ♯1–4, ♯3–3) in Table 2 were electrophoresed on 2% agarose gel and stained in ethidium bromide. White arrows indicate the target transcripts of PML-RARα *bcr1* and *bcr3* (sp; *bcr1*: 639 bp, *bcr3*: 164 bp, TM; *bcr1*: 127 bp, *bcr3*: 145 bp). Black-tipped arrows indicate alternative spliced bands. No arrow bands indicate nonspecific bands. sp: sp-RQ-PCR using LightCycler; TM: TaqMan assay using ABI7700; M: size marker.

also yielded some nonspecific bands. ♯3–3 also showed the TM assay had some nonspecific bands. In the same manner, 31 samples from non-APL patients were tested with the sp-RQ-PCR and TM assay. Although all samples, which tested negative in the nested PCR, were also negative in the sp-RQ-PCR, four of them (13%) were identified as positive by the TM assay, that is, positive cases of Major-BCR/ABL, AML1/MTG8, E2A/PBX1, and WT1. All these amplification products were also checked by electrophoresis, which verified the presence of artifacts in the cases detected by the TM assay, and the subsequent sequencing analysis confirmed none of them corresponded to PML-RARα (data not shown).

DISCUSSION

Although quantification procedures of PML-RARα transcript based on TaqMan system using multiple primers have come into use, some studies have reported that these approaches can give rise to false-positive results (21,22) and we experienced the same problem when performing these reported assays. We therefore attempted to establish a RQ-PCR assay that can not only avoid false-positive results but also detect various types of PML-RARα fusion transcript with only a single forward primer (sp-RQ-PCR). In the study presented here, we compared the sp-RQ-PCR with LC and TM assay using three primer pairs with ABI prism 7700.

For the sp-RQ-PCR, the standard curves generated by four plasmid cDNAs obtained from different types of isoforms (Fig. 1) were reproducible in successive assays, yielding very consistent C_T for each one of the points. The standard straight line fitting five dilution points showed a very high correlation coefficient (0.99) with the linearity over five orders of magnitude, and the medium slope value of the standard curve (−3.6) was similar to that for optimum PCR amplification (−3.3) (Fig. 3). These results suggest that the sp-RQ-PCR could simultaneously detect *bcr1*, *bcr2*, and *bcr3* isoforms as well as alternative spliced forms with equal specificity. Some studies have reported clinical relevance of the specific type of PML-RARα fusion transcript expressed in an individual (10–15). It is therefore essential to detect and differentiate long, short, or variant forms as well as alternative spliced forms. Although the sp-RQ-PCR has sufficient capability to detect the PML-RARα transcript, complementary use of gel electrophoresis of the PCR products is recommended to verify the various types of PML-RARα fusion transcripts, including alternative spliced forms and nonspecific bands.

The sp-RQ-PCR could detect one single PML-RARα positive cell among between 10^4 (1 μg total RNA) and 5×10^4 (cDNA) negative cells, which was similar to the result for the TM assay, but somewhat lower than for the nested PCR and a procedure that reportedly detected one positive cell among 10^5 negative cells (26). However, our results were based on recommendations determined in a study using the RQ-PCR for TaqMan in which there were approximately 10^4 copies of the long-form fusion transcript per 1 μg of total RNA contained in the NB4 cell line (27). The CVs of the inter- and intra-assays were 2.0–3.6% and 1.4–3.3% for the long and short form, respectively, demonstrating that the sp-RQ-PCR was sensitive and reliable enough for the detection of PML-RARα transcript.

To compare the absolute amounts of PML-RARα transcript identified by the sp-RQ-PCR and TM assay, 39 samples from 15 APL patients and 31 samples from non-APL leukemia patients were tested. Concordance between two assays was high, with a correlation coefficient higher than 0.9. However, when C_T values more than 40 were not considered PCR positive, in two cases, PML-RARα transcript was detected in the TM assay, but not in the sp-RQ-PCR or nested PCR. Electrophoresis of their amplification products discovered an artifact in the TM assay, which sequencing analysis proved not to correspond to PML-RARα (Fig. 4, ♯1–4). For high expression levels of PML-RARα transcripts, both assays detected alternative spliced bands besides the long-form band (Fig. 4, ♯1–1), and the TM assay yielded some nonspecific bands as well (♯1–1, ♯3–3). Additionally, in the 31 samples from non-APL patients, four (13%) were detected in the TM assay, but not in the sp-RQ-PCR, as confirmed by sequencing analysis. Although the EAC (A Europe Against Cancer Program) has reported that the C_T values in the false-positive samples were always more

than 30 cycles and most of the time higher than 35 cycles (22), the potential for false-positive results associated with multiple primers must not be overlooked.

Although PML-RARα was one of the first targets used for MRD detection in clinical studies, a few studies, most of which have been developed for the TM system, have used the RQ-PCR for PML-RARα (22,28,29). This TaqMan technology by ABI exploits Taq 5′ exonuclease activity, which hydrolyzes the hybridized TaqMan probe during the elongation step. This hydrolysis then releases the labeled reporter dye from the quencher dye, which is conjugated to the probe. As a result, the extension of the PCR products can be tracked by monitoring the fluorescence intensity of the light emitted by the laser-excited, released reporter molecules (30). However, to achieve 5′ nuclease activation, an elongation–annealing time of at least 1 min is generally required. LC technology, on the other hand, may provide additional methodological advantages, such as a further increase in PCR speed that results from the fast ramp rate during heating and cooling. This, combined with the Hyb-Probe technology, allows completion of the assay in only 45 min. Furthermore, the fast ramp times and the brevity of the annealing and elongation steps improve the specificity and yield very clean PCR products, indicating that no false-positive product are detected, and no background signal is subtracted. Added to these mechanical differences, the TM assay requires a primer and probe set for each of the three isoforms, which is likely to generate some nonspecific products including false-positive ones. This means that the more oligonucleotides are in the reaction mix, the higher the probability is of the occurrence of unspecific bindings, including polymerization of primers and probes. These results further suggest that the difference between the sp-RQ-PCR and TM assay depends on not only the mechanical differences between LC and TM (ABI7700) but also the number of oligonucleotides such as primers and probes. The sp-RQ-PCR is basically an RQ-PCR that uses two forward primers, one for the long and one for the short form (23). Here, we confirmed that optimization of the PCR conditions in LC enabled a primer (*bcr3*) for the short form to detect the long form without a long-form primer (*bcr1*).

At present, most of the published TM and LC protocols for PML-RARα use multiple primers, whereas the sp-RQ-PCR is currently the only protocol that allows for quantification of PML-RARα transcripts with a single primer pair. The findings of our study demonstrate that our sp-RQ-PCR is sensitive and specific, can simultaneously detect all PML-RARα isoforms without false-positive results, and has the advantage of accomplishing the entire assay in only 45 min. These results suggest the sp-RQ-PCR may constitute a highly reliable tool for detection of PML-RARα transcript. Further clinical studies involving APL patients are required to determine whether the sp-RQ-PCR can also provide relevant quantitative information before, during, and after therapy.

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