# Comprehensive analysis of CBFβ-MYH11 fusion transcripts in acute myeloid leukemia by RT-PCR analysis

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# Comprehensive Analysis of *CBFβ-MYH11* Fusion Transcripts in Acute Myeloid Leukemia by RT-PCR Analysis

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***CBFβ-MYH11* fusion transcripts are expressed in acute myeloid leukemias of the M4Eo subtype. Patients who express *CBFβ-MYH11* fusion transcripts respond favorably to high-dose chemotherapy and are generally spared allogeneic bone marrow transplantation. Hence it is important to identify this fusion in all patients with acute myeloid leukemia M4Eo leukemia. The fusion can be detected by cytogenetics, fluorescence *in-situ* hybridization (FISH), or by molecular analysis with RT-PCR. Multiple fusion transcripts arising as a result of various breakpoints in the *CBFβ* and *MYH11* have been identified. In this report we describe a comprehensive RT-PCR assay to identify all known fusion transcripts and provide an algorithm for molecular analysis of *CBFβ-MYH11* fusions from patient specimens. Further, identification of the fusion transcript by such an assay would help in the diagnosis and follow up of patients with cryptic inversion 16 translocations (such as patient 2 in this report) not detected by standard cytogenetics or FISH and for rational design of probes for quantitative analysis by real-time PCR. *(J Mol Diagn 2004, 6:22–27)***

*CBFβ-MYH11* fusion transcripts are expressed in acute myeloid leukemias of the M4Eo subtype and occur as a result of inversion 16 (p13q22) or t(16;16) chromosomal abnormalities.[1–4] These leukemias have a relatively favorable response to high-dose chemotherapy and such patients are generally spared allogeneic bone marrow transplantation.[5,6] Thus detection of *CBFβ-MYH11* fusion transcripts at diagnosis is important for making therapeutic decisions.

The fusion transcripts involve the *CBFβ* gene on 16q22 and the *MYH11* gene on 16p13. CBFβ together with CBFα subunit forms the core binding factor complex. The DNA binding activity of CBFα is stabilized by dimerizing with CBFβ.[7] The resulting CBFα-CBFβ complex has transcriptional activity and recognizes the core regulatory sequence TGTGGT, present in several cellular and viral promoters and enhancers as well as in genes expressed in hematopoietic cells.[8] Three genes encode CBFα (*RUNX2, RUNX1* and *RUNX3*), whereas a single gene codes for CBFβ. *CBFα* expression is limited to hematopoietic system in adults whereas *CBFβ* appears to be ubiquitously expressed.[7] Knock-out and knock-in studies show that *CBFα* and *CBFβ* expression is absolutely essential for normal hematopoiesis and differentiation.[9,10] *MYH11* codes for the smooth muscle myosin heavy chain. The repeated coiled coil structure of the tail domain of MYH11 may result in dimerization of the *CBFβ-MYH11 f*usion protein and lead to alterations in transcriptional regulation. The *CBFβ-MYH11* fusion protein is believed to bind and sequester CBFα resulting in dysfunctional transcription and leukemogenesis.[7]

Three different techniques are used to identify *CBFβ-MYH11* fusions: cytogenetics, fluorescence *in-situ* hybridization (FISH), and molecular analysis using RT-PCR. The breakpoints in the *CBFβ* and *MYH11* are variable and thus testing by all three methods may be necessary.[8,11] Some reports suggest that RT-PCR analysis is more sensitive than standard cytogenetic or FISH analysis to detect *CBFβ-MYH11* fusions.[12–14] Standard cytogenetics and FISH generally have a sensitivity of 1 to 5%, depending on the number of cells examined and the type of probe used, respectively. In most cases cytogenetic and molecular analysis correlate with each other, but acute myeloid leukemia (AML) M4 Eo patients who are negative for inv(16) by standard cytogenetics but positive for fusion gene transcripts by RT-PCR have been described.[12–16] Cytogenetic analysis is still required to obtain a complete karyotype in every patient with AML because of the well-described prognostic significance of the karyotype in this disease.[17] In addition, cytogenetic analysis helps to follow evolutionary changes in karyotype that may be related to overall prognosis.

Molecular analysis by RT-PCR is a rapid and sensitive method for analyzing the expression of *CBFβ-MYH11* fusion transcripts. Breakpoint locations in the *CBFβ* and *MYH11* can also be determined by molecular analysis.

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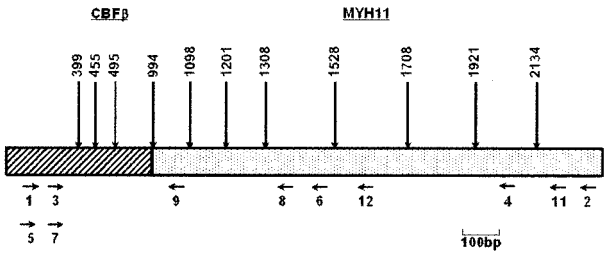


**Figure 1.** Primer locations in relation to known breakpoints in the *CBFβ* and *MYH11* cDNA.

This information might have prognostic significance, although such significance has not yet been established. There are eight known breakpoints in the *MYH11* and three known breakpoints in the *CBFβ* (Figure 1). The breakpoints in the *MYH11* occur at nucleotide (nt) positions 994 (Type E), 1098 (Type H), 1201 (Type D or G), 1308 (Type X), 1528 (Type C), 1708 (Type B), 1921 (Type A), and 2134 (Type S) compared with *MYH11* GenBank sequence D10667.1 GI:532875.[8] The breakpoints in the *CBFβ* occur at nt positions 495 [most common], 455[18] or 399[8,11,19] compared with GenBank sequence AF294326.1 GI:9885832. The most common fusion is between nt 495 of the *CBFβ* and nt 1921 of the *MYH11* sequence. This fusion accounts for up to 88% of all *CBFB-MYH11* chimeric transcripts. Fusions between nt 495 of the *CBFβ* sequence and nt 994 or 1201 of the *MYH11* sequence account for about 10% of cases. The remaining breakpoints account for less than 2% of cases. However, one can never predict a particular breakpoint prospectively and hence there is a need for a comprehensive molecular assay to detect all known fusions from clinical specimens in a systematic manner. In addition, alternative splicing of *CBFβ-MYH11* fusion transcripts has been reported.[4] Further, we are unaware of any published report of the presence of *CBFβ-MYH11* fusion transcripts in blood from healthy individuals by standard RT-PCR assays.

To the best of our knowledge, published reports to date have used only a few primer sets to identify the breakpoints and fusions resulting from them. False-negative results can occur if the breakpoints lie outside the ability of detection with a particular primer set. The problem can be overcome by using multiple primer sets to target all of the known breakpoints in the two genes. A disadvantage to using multiple primer sets is the need to run multiple reactions on every sample, which may be difficult if the amount of sample RNA is limiting. Multiplexing different primers in the same reaction is an option, but the amplification products obtained by multiplexing primer sets may generate a complicated pattern of differently sized products that may be hard to interpret. Also, RNA degradation, a potential problem in clinical samples, will require that the amplicon sizes be short to avoid false negatives. In this report, we propose an optimized algorithm that uses multiple primer pairs in different reactions in a stepwise manner to detect all known breakpoints in both *CBFβ* and *MYH11*. We further illustrate the utility of such an algorithm to detect *CBFβ-MYH11* fusion transcripts in two patients with AML M4Eo.

**Table 1.** Primer Sequences

| Primer | Sequence, 5′ > 3′ |
|---|---|
| Inv16-1, sense | GCAGGGAGAACAGCGACAAACA |
| Inv16-2, anti-sense | TCTGGAGGCACGGGCATC |
| Inv16-3, sense | ATGGGCTGTCTGGAGTTTGAT |
| Inv16-4, anti-sense | GCGCCTGCATGTTGACTT |
| Inv16-5, sense | GCAGGGAGAACAGCGACAAA |
| Inv16-6, anti-sense | TCTTCCAGAGCTTCCACGGT |
| Inv16-7, sense | ATGGGCTGTCTGGAGTTTGA |
| Inv16-8, anti-sense | TGGCGCAGCTTCGTAGACAC |
| Inv16-9, anti-sense | GGCCAGGTCTGCGTTCTC |
| Inv16-11, anti-sense | AGCCTGCAGTTTGCGTAGCTG |
| Inv16-12, anti-sense | GAGTTGCCGCTGGTTGTC |

## Materials and Methods

### Samples and RNA Extraction

RNA was extracted from blood or bone marrow samples and from the ME-1f2 cell line with the RNeasy Mini kit according to the manufacturer's directions (Qiagen, Valencia, CA). Contaminating genomic DNA was eliminated by on-column digestion with DNase I (Qiagen). The ME-1f2 cell line was a kind gift from Dr. Ikulya Sakai, First Department of Internal Medicine, Ehime University, Ehime, Japan.[20] ME-1f2 was maintained in RPMI 1640 with 10% fetal calf serum (FCS) and antibiotics in a humidified atmosphere containing 5% $CO_2$. This cell line is a subline of ME-1, which was established from a patient with AML M4Eo. ME-1f2 harbors the inversion 16 chromosomal abnormality and expresses Type A *CBFβ-MYH11* fusion transcripts.

### Primer Design

Primers were designed using GenBank sequences D10667.1 GI:532875 (*CBFβ*) and AF294326.1 GI:9885832 (*MYH11*) as templates and were optimized using Oligo Version 6.0 software (Molecular Biology Insights, Inc., Cascade, CO). The primer sequences are listed in Table 1. The locations of the primers in relation to the breakpoints in *CBFβ* and *MYH11* are shown in Figure 1.

### RT-PCR Analysis

Molecular analysis was performed by reverse transcription followed by nested PCR amplification. All amplifications were performed in a MJ Research PTC 200 DNA Engine thermocycler (MJ Research, Watertown, MA). For the first-round reaction, 1 μg of RNA was reverse transcribed and amplified using the One Step RT-PCR kit (Qiagen). Each 50 μl reaction contained 1X RT-PCR buffer with 2.5 mmol/L $MgCl_2$, 0.4 mmol/L of each dNTP, 30 pmols of forward and reverse primers, 1X Q solution, 10U RNase inhibitor (Promega, WI), and 2 μl of an enzyme mix that contained reverse transcriptase and HotStarTaq DNA polymerase. Reverse transcription was at 48° for 1 hour, followed by 95° for 15 minutes to inactivate reverse transcriptase and simultaneously activate HotStarTaq DNA polymerase. This was followed by 40 cycles

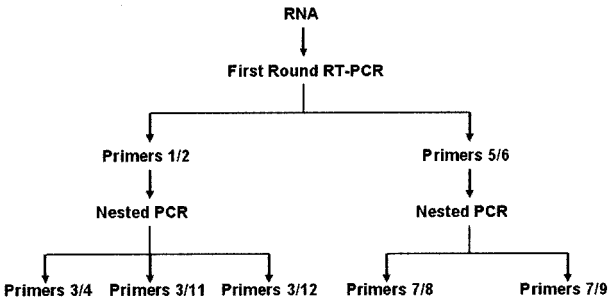


**Figure 2.** Algorithm for the comprehensive analysis of *CBFβ-MYH11* fusion transcripts.

of PCR amplification. Each cycle was 92° for 30 seconds, 61° for 30 seconds, and 72° for 35 seconds. A final extension step was performed at 72° for 10 minutes.

Nested PCR amplification was performed with the HotStarTaq DNA polymerase kit (Qiagen). Each 50 μl reaction contained 1X PCR buffer containing 1.5 mmol/L $MgCl_2$, 0.2 mmol/L of each dNTP (Sigma, St. Louis, MO), 20 pmols of forward and reverse primers and 0.5 μl (2.5U) of HotStarTaq DNA polymerase enzyme. One μl of a 1:10 dilution of the first-round product was used as template. Each reaction was heated at 95° for 15 minutes to activate the HotStarTaq enzyme and to denature the template and this step was followed by 33 cycles of PCR amplification. Each cycle was 92° for 30 seconds, 64° for 28 seconds, and 72° for 30 seconds. A final extension step was at 72° for 10 minutes.

### *Gel Electrophoresis*

Ten μl of the nested PCR product was electrophoresed on a 2% agarose gel (E-Gel, Invitrogen, Carlsbad, CA). The gel was photographed under UV transillumination with the AlphaImager system (AlphaInnotech, San Leandro, CA).

### *Cloning and Sequencing*

Nested PCR products were cloned into pCRII TOPO vector (Invitrogen) for good quality sequencing results and to generate positive-control plasmids and RNA for further use in the laboratory. TOP10 cells were transformed and plated on to LB/amp plates with blue/white screening. Representative clones were sequenced by the DNA Sequencing Core Facility at the University of Illinois at Chicago. Sequence analysis was performed with the BLAST program (www.ncbi.nih.gov).

## *Results*

### *RT-PCR Analysis*

The algorithm in Figure 2 was applied to RNA samples from two patients with AML M4Eo and the cell line ME1-f2. In the initial step, first-round amplification was performed with primers 1/2 followed by nested reactions with primers 3/4, 3/11, or 3/12. Patient 1 showed products of

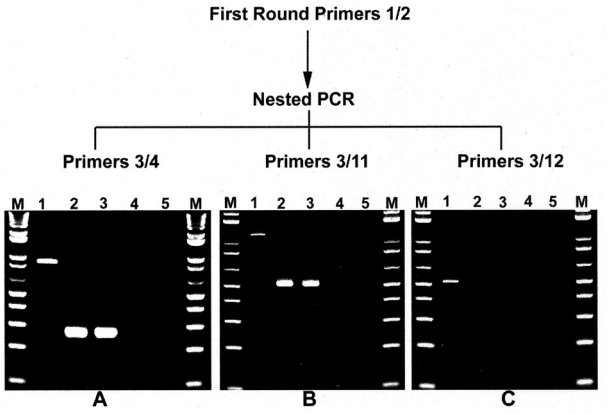


**Figure 3.** First-round RT-PCR with primers 1/2 followed by nested PCR with primers 3/4 (**A**), 3/11 (**B**) or 3/12 (**C**). M-1Kb Plus molecular weight marker (Invitrogen). **Lane 1**, patient 1; **lane 2**, patient 2; **lane 3**, ME-1f2 cells; **lane 4**, HL-60; **lane 5**, water control.

892 bp, 1134 bp, and 504 bp with nested primers 3/4, 3/11, or 3/12, respectively (Figure 3A to C, lane 1). Patient 2 and ME-1f2 cells showed 268-bp and 510-bp products with nested primers 3/4 (Figure 3A, lanes 2 and 3) and 3/11, respectively (Figure 3B, lanes 2 and 3). No product was obtained from patient 2 and ME1-f2 cells when the nested reaction was performed with primers 3/12 (Figure 3C, lanes 2 and 3).

In the next step, a separate first-round amplification was performed with primers 5/6 followed by nested reactions with primers 7/8 or 7/9. Patient 1 showed a 197-bp product with nested primers 7/8 (Figure 4A, lane 1). No product was obtained from patient 1 with primers 7/9 (Figure 4B, lane 1). Also, no products were obtained from patient 2 and ME-1f2 cells with primers 7/8 or 7/9 (Figure 4, A and B, lanes 2 and 3).

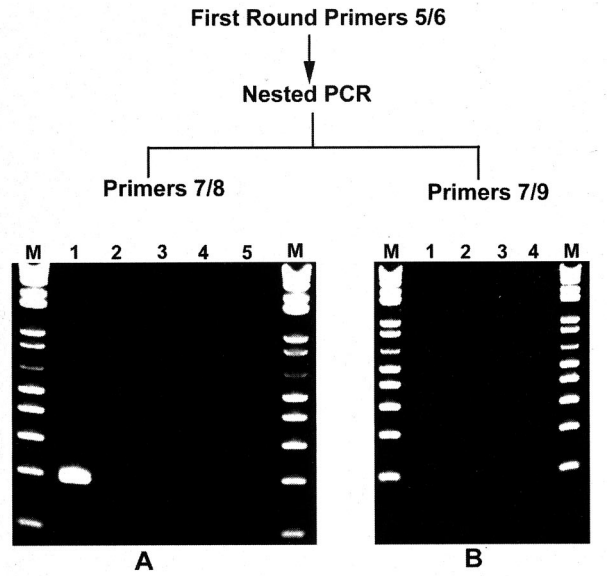


**Figure 4.** First-round RT-PCR with primers 5/6 followed by nested PCR with primers 7/8 (**A**) or 7/9 (**B**). M-1Kb Plus molecular weight marker (Invitrogen). **A: Lane 1**, patient 1; **lane 2**, patient 2; **lane 3**, ME-1f2 cells; **lane 4**, HL-60; **lane 5**, water control. **B: Lane 1**, patient 1; **lane 2**, patient 2; **lane 3**, ME-1f2 cells; **lane 4**, water.

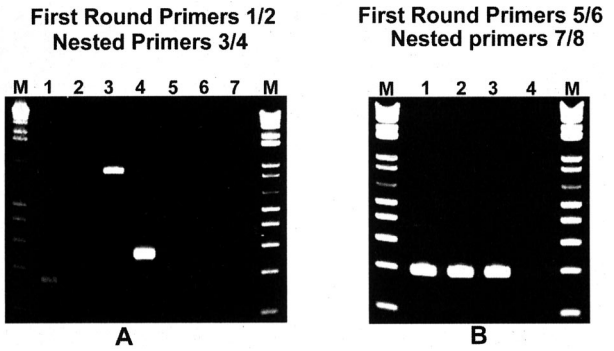


**Figure 5.** Effect of RNA degradation on amplification. **A:** Analysis with primers 1/2 followed by primers 3/4: **lanes 1** and **2**, two previous RNA samples from patient 1; **lane 3**, most recent sample from patient 1 (same as in **lane 1**, Figure 3**A**); **lane 4**, ME-1f2 cells; **lane 5**, HL-60; **lanes 6** and **7**, water controls. **B:** Analysis with primers 5/6 followed by primers 7/8. **Lanes 1** and **2**, two previous samples from patient 1; **lane 3**, most recent sample from patient 1 (same as lane 1, Figure 4A); **lane 4**, water control.

RNA from HL-60 cells did not amplify with any primer set. Unidirectional workflow in the laboratory was strictly followed and great care was taken to prevent contamination. Water-only reactions did not show any amplification products, diminishing the likelihood of cross-contamination. Further, peripheral blood or bone marrow-derived RNA from 32 cases of unrelated leukemia and 20 healthy individuals did not show amplification products with any of the primer sets.

Two previous RNA samples from patient 1 (Figure 5A, lanes 1 and 2) did not show any specific amplification product with primers 1/2 followed by a nested reaction with primers 3/4. A few non-specific products of incorrect size were seen in the sample analyzed in lane 1. However, the same two RNA samples amplified with primers 5/6 followed by a nested reaction with primers 7/8 (Figure 5B, lanes 1 and 2). The presence of amplifiable RNA was confirmed by prior analysis of tyrosine kinase mRNA (ABL) expression. The ABL primers amplified a product of 315 bp (data not shown) in the two samples. Sequencing results suggested that partial RNA degradation in the previous samples from patient 1 was the likely cause of amplification failure with primer sets 1/2 and 3/4. In this patient, first-round primers 1/2 are expected to amplify a 1327-bp product in contrast to a 498-bp product from primers 5/6. Thus if RNA degradation reduced the size of intact transcripts, primers 5/6 would have had a better chance of generating an amplified product compared to primers 1/2. Partial RNA degradation would also explain the positive ABL result (315-bp product) and a negative result with primer sets 1/2 and 3/4. Hence false-negative results occurred with primers 1/2 and 3/4 in the two previous samples from patient 1. These samples were in fact positive for fusion transcripts as detected by primers 5/6 and 7/8. The built-in redundancy in the assay assures against false-negative results and represents a significant advantage of this analysis protocol.

## *Sequence Analysis*

The pattern of bands in patient 1 suggested a proximal 5′ breakpoint in *MYH11*. The 197-bp product (generated by primers 5/6 and 7/8) that was sequenced showed that the breakpoint in *CBFβ* was at nt 399 and the breakpoint in *MYH11* was at nt 1201. This represents a "Type D" breakpoint in *MYH11* (reference 11 and Figure 1). Individual breakpoints at nt 399 in *CBFβ* and at nt 1201 in *MYH11* have been reported before.[8,11,17] However, to the best of our knowledge, there is only one previously published report of a single case of a fusion between nt 399 of *CBFβ* and nt 1201 of *MYH11*.[8] Patient 1 in our report is thus the second case of *CBFβ-MYH11* fusion occurring as a result of a breakpoint at nt 399 in *CBFβ* and nt 1201 in *MYH11*. Sequence analysis of the 268-bp product from patient 2 and ME-1f2 cells (generated by primers 1/2 and 3/4) showed that the breakpoint in *CBFβ* was at nt 495 and in *MYH11* was at nt 1921. This represents a typical "Type A" fusion transcript that is seen in almost 85 to 88% of cases with *CBFβ-MYH11* fusions. Interestingly, cytogenetic and FISH analysis failed to detect inversion 16 or t(16;16) inpatient 2 where as ME-1f2 cells and leukemic cells from patient 1 showed the inversion 16 abnormality by cytogenetics. Thus, the only evidence of *CBFβ-MYH11* fusion inpatient 2 was from molecular analysis by RT-PCR.

## *Discussion*

In this report, we describe a comprehensive assay to identify *CBFβ-MYH11* fusion transcripts. The assay utilizes two first-round RT-PCR reactions and five nested reactions and can be completed within 24 hours after RNA extraction. Because only two first-round RT-PCR reactions are performed, the assay can be performed with as little as 2 μg of total RNA (1 μg/reaction). Our

**Table 2.** Expected Amplicon Sizes Generated by the Respective Primer Pairs*

| | | CBF 495 | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | MYH11 | 2134 | 1921 | 1708 | 1528 | 1308 | 1201 | 1098 | 994 |
| Primers 1/2 | 1st round | 490 | 703 | 918 | 1096 | 1318 | 1423 | 1526 | 1630 |
| Primers 3/4 | Nested | NA | 268 | 481 | 666 | 881 | 988 | 1091 | 1195 |
| Primers 3/11 | Nested | 297 | 510 | 723 | 903 | 1123 | 1230 | 1333 | 1437 |
| Primers 3/12 | Nested | NA | NA | NA | 273 | 493 | 600 | 703 | 807 |
| Primers 5/6 | 1st round | NA | NA | NA | NA | 487 | 594 | 697 | 801 |
| Primers 7/8 | Nested | NA | NA | NA | NA | 186 | 293 | 396 | 500 |
| Primers 7/9 | Nested | NA | NA | NA | NA | NA | NA | NA | 192 |

NA, Not amplified.
* Amplicons are shorter by 40 bp or 96 bp, if the breakpoints in *CBFβ* occur at nt 455 or 399 respectively.

assay utilizes a nested format with a larger number of primer sets and has distinct advantages over previously published reports.[8,11] First, the primer sets cover all currently known breakpoints in the *CBFβ* and *MYH11* genes.[11] Expected product sizes generated with the primer sets from all potential fusions are shown in Table 2. Hence, accurate identification of the breakpoints in *CBFβ* and *MYH11* genes is possible. The protocol does use multiple primer sets in separate reactions, making the assay seem a bit tedious. It is certainly possible to assay the most common fusion transcript first (Type A, primers 1/2, and nested primers 3/11) and then use the other primer sets if the patient is negative for Type A transcripts. However, the purpose of this report is to provide a comprehensive assay to detect all known fusion transcripts arising from multiple breakpoints in AML M4 Eo cases. The strength of the comprehensive format is that more than one nested reaction performed concurrently produces a product from the same fusion transcript (albeit of a different size, Table 2). This approach significantly improves specificity and sensitivity and confers a high level of confidence in interpreting results without the need for further confirmation by probe hybridization and detection. Such an approach also improves turn-around times for clinical samples because all reactions are performed simultaneously. Multiplexing different primer pairs was considered but such an approach resulted in primer-dimer formation and decreased the efficiency of specific amplification.

Second, the product sizes generated from the primer sets are such that reasonably preserved RNA can still be analyzed and false-negative results can be avoided. This was illustrated by results obtained from two previous samples from patient 1 (Figure 5). RNA can degrade if sufficient care is not taken to preserve RNA integrity. The two previous samples from patient 1 were negative for fusion transcripts when analyzed with primers 1/2 and 3/4. However the same samples were positive when re-analyzed with primers 5/6 and 7/8. These results suggest that RNA degradation led to false-negative results with primers 1/2 and 3/4 as discussed in the Results section. Thus the assay is capable of analyzing accurately, even suboptimal RNA samples, although completely degraded RNA would be unsuitable for any analysis. In our opinion, the built-in redundancy of the assay represents a distinct advantage.

Third, some previous reports have used post-PCR steps such as oligonucleotide hybridization after a single round of RT-PCR to confirm identity of the amplicons.[8] The algorithm presented in this report utilizes more than one set of nested amplification reactions to identify fusion transcripts in every case thereby ensuring a high degree of specificity, obviating the routine need for confirmation by additional probe hybridization. The assay can reliably detect *CBFβ-MYH11* fusion transcripts in as little as 10 pg of ME-1f2 cells when diluted into 1 $\mu$g of negative HL-60 RNA and hence can be used to monitor minimal residual disease qualitatively. This sensitivity was achieved only with a nested protocol. The sensitivity of a single round of amplification was about 100-fold lower than the nested protocol. Thus the protocol ensures adequate specificity and increased sensitivity without the need for post-PCR confirmatory testing. However, sequence confirmation may still be necessary when amplicons of unexpected sizes are found either from fusion transcripts arising from hitherto undescribed breakpoints or by alternative splicing. More extensive studies are necessary before the frequency of the need for sequencing can be determined.

Although only two cases were presented in this report, we believe that the assay is robust enough to comprehensively identify all known *CBFβ-MYH11* fusion transcripts. Further, in patient 2, routine cytogenetic analysis and FISH failed to reveal the inversion 16 abnormality and molecular analysis by RT-PCR was the only method that detected *CBFβ-MYH11* fusion transcripts.[16] Such cases have been reported previously and probably arise from small insertions that are below the detection ability of standard cytogenetics and FISH.[12–15] We have also not observed any false-positive results with unrelated RNA specimens from patients with leukemias other than AML M4Eo.

Although the protocol outlined in this report can be used for qualitative detection of minimal residual disease in patients with AML and inv(16), quantitative analysis of fusion transcripts is necessary for determining the kinetics of minimal residual disease.[21] However, before quantitative analysis can be performed, it is critical to determine the type of fusion transcript by qualitative analysis so that appropriate primers and probes can be chosen for quantification. This is necessary because of length restrictions for reliable amplification by real-time PCR systems. For example, primers and probes for the detection and quantification of the Type A breakpoint in *MYH11* would be inappropriate for the detection of the Type E or D breakpoints due to length restrictions. Similarly primers and probes optimized for Type E or D breakpoints based on short amplicon sizes (less than 250 bp) would be inappropriate to detect the Type A breakpoint. Hence, we believe that qualitative analysis with a comprehensive assay as the one reported herein should be the first step in the analysis of any patient suspected of having *CBFβ-MYH11* fusion bearing leukemia. This information will assist in the rational choice of the appropriate primer-probe combinations for quantitative analysis by real time PCR and also help to determine the prognostic significance of various fusion transcripts in different patients.

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