Technical Advance

Rapid Simultaneous Amplification and Detection of the MBR/JH Chromosomal Translocation by Fluorescence Melting Curve Analysis

Sandra D. Bohling,* Thomas C. King,[†]
Carl T. Wittwer,* and
Kojo S. J. Elenitoba-Johnson*

From the Departments of Pathology,* University of Utah Health Sciences Center and ARUP Institute for Clinical and Experimental Pathology, Salt Lake City, Utah, and the Roger Williams Medical Center[†] and Brown University School of Medicine, Providence, Rhode Island

Polymerase chain reaction (PCR) amplification and product analysis for the detection of chromosomal translocations, such as the t(14;18), has traditionally been a two-step process. PCR product detection has generally entailed gel electrophoresis and/or hybridization or sequencing for confirmation of assay specificity. Using a microvolume fluorimeter integrated with a thermal cycler and a PCR-compatible double-stranded DNA (dsDNA) binding fluorescent dye (SYBR Green I), we investigated the feasibility of simultaneous thermal amplification and detection of MBR/JH translocation products by fluorescence melting curve analysis. We analyzed DNA from 30 cases of lymphoproliferative disorders comprising 19 cases of previously documented MBR/JH-positive follicle center lymphoma and 11 reactive lymphadenopathies. The samples were coded and analyzed blindly for the presence of MBR/JH translocations by fluorescence melting curve analysis. We also performed dilutional assays using the MBR/JH-positive cell line SUDHL-6. Multiplex PCR for MBR/JH and β -globin was used to simultaneously assess sample adequacy. All (100%) of the 19 cases previously determined to be MBR/JH positive by conventional PCR analysis showed a characteristic sharp decrease in fluorescence at $\sim 90^\circ\text{C}$ by melting curve analysis after amplification. Fluorescence melting peaks obtained by plotting the negative derivative of fluorescence over temperature ($-dF/dT$) versus temperature (T) showed melting temperatures (T_m) at $88.85 \pm 1.15^\circ\text{C}$. In addition, multiplex assays using both MBR/JH and β -globin primers yielded easily distinguishable fluorescence melting peaks at

$\sim 90^\circ\text{C}$ and 81.2°C , respectively. Dilutional assays revealed that fluorescence melting curve analysis was more sensitive than conventional PCR and agarose gel electrophoresis with ultraviolet transillumination by as much as 100-fold. Simultaneous amplification and fluorescence melting curve analysis is a simple, reliable, and sensitive method for the detection of MBR/JH translocations. The feasibility of specific PCR product detection without electrophoresis or utilization of expensive fluorescently labeled probes makes this method attractive for routine molecular diagnostics. (*Am J Pathol* 1999, 154:97-103)

The t14;18(q32;q21) chromosomal translocation is the most common specific recurrent karyotypic abnormality in non-Hodgkin's lymphomas (NHLs). It is detectable in up to 85% of cases of follicle center lymphoma (FCL) and up to 30% of diffuse large-B-cell lymphomas.^{1,2} This translocation juxtaposes the bcl-2 gene on chromosome 18q21 to one of the immunoglobulin heavy chain joining region (JH) sequences on 14q32 and results in overexpression of bcl-2 protein with consequent increased longevity of the follicle center cells.^{3,4} Approximately 70% of the breakpoints on chromosome 18 are clustered within a 150-bp segment located in the 3' untranslated region of exon 3 of the bcl-2 gene designated as the major breakpoint region (MBR).⁵ The clustering of the breakpoints on chromosome 18 and the high degree of sequence homology between the 3' portions of the JH segments have permitted the development of highly sensitive polymerase chain reaction (PCR)-based assays that have facilitated the clinical diagnosis⁶ and monitoring of minimal residual disease in patients with FCL.⁷

PCR assays have traditionally entailed a two-step procedure comprising an initial amplification reaction in a thermal cycler, followed by PCR product analysis in a

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Address reprint requests to Dr. Kojo S. J. Elenitoba-Johnson, Division of Anatomic Pathology, University of Utah Health Sciences Center, 50 North Medical Drive, Salt Lake City, UT 84132. E-mail: kojo.elenitobaj@path.med.utah.edu.

separate process.^{6,8} Analysis of the PCR product has generally involved size fractionation by gel electrophoresis and product visualization by ultraviolet (UV) transillumination of ethidium-bromide-stained gels or by radioisotopic detection. More recently, MBR gene rearrangements have been detected using fluorescently labeled primers during amplification, followed by a separate process of product detection by flow cytometric analysis⁹ or automated electrophoresis-based DNA sizing technology.¹⁰

PCR amplification with simultaneous amplicon analysis can be accomplished by incorporating a double-stranded DNA (dsDNA)-specific dye into amplification reactions carried out in glass capillary tubes.¹¹ Fluorescence is monitored once per cycle after product extension, with the increase in fluorescence related to product accumulation. As the fluorescent dye indiscriminately detects all dsDNA, distinction of the PCR amplicon from the template, primer dimers, and other nonspecific dsDNA within the reaction solution is necessary. As the melting characteristics of any segment of dsDNA are specified by its GC content, length, and nucleotide sequence, determination of product identity can be achieved by virtue of its melting temperature.¹²

In this report, we present the detection of MBR/JH translocations using a rapid thermal cycler integrated with a microvolume fluorimeter that affords simultaneous thermal amplification and product identification by fluorescent melting curve analysis in less than 45 minutes. The product identity was confirmed as MBR/JH sequence in all of the 19 cases determined to be positive for the MBR/JH translocation product by fluorescence PCR. Our findings indicate that this method is a faster and more sensitive means of detection of MBR/JH translocations than conventional methods involving gel electrophoresis and has potential for improving turn-around time in the diagnostic laboratory.

Materials and Methods

DNA Samples

A total of 30 DNA samples previously assessed for the presence of the MBR/JH translocation by conventional PCR⁶ were randomly pulled from the files of the molecular diagnostics laboratories of the Roger Williams Medical Center, Providence, RI, and ARUP Laboratories, Salt Lake City, UT. These samples were assigned arbitrary code numbers to ensure unbiased evaluation and comparison with the results obtained using fluorescence melting-curve-based detection of the PCR products. These samples were composed of 19 known MBR/JH-positive cases, one of which was positive for the MBR/JH translocation by conventional PCR and contained Epstein-Barr virus (EBV) DNA sequences. We also examined 11 MBR/JH-negative cases, 6 of which were known to be positive for EBV DNA by PCR. DNA was also extracted from the well characterized SUDHL-6 cell line with a known MBR/JH6 translocation for dilution assays.

Conventional PCR Analysis

PCR analysis for MBR/JH translocations was performed as previously described.^{6,8,13} PCR assays designed to detect EBV DNA¹⁴ were performed to exclude the errant detection of EBV sequences by MBR/JH PCR as previously described.¹⁵ PCR products were subjected to electrophoresis in a 1.5% agarose gel (SeaKem LE agarose, FMC Bioproducts, Rockland, ME) containing 1X Tris-buffered ethanolamine and 0.5 μ g/ml ethidium bromide. Amplification reactions containing a discrete band at the expected electrophoretic migration were considered to be positive (80 to 300 bp), using the Biomarker Low DNA ladder with band sizes of 1000, 700, 525/500, 400, 300, 200, and 100 bp as the DNA size marker (Bioventures, Murfreesboro, TN). The sensitivity of the conventional MBR/JH PCR with subsequent gel electrophoresis method was assessed using an initial concentration of 50 ng/ μ l of the MBR/JH-positive SUDHL-6 cell line DNA with serial dilutions of 1:2, 1:5, 1:10, 1:50, 1:100, 1:200, 1:300, 1:400, 1:500, and 1:1000 into placental DNA.

PCR and Fluorescence Melting Curve Analysis

Rapid cycle PCR analysis was performed with paired oligonucleotide primers specific for the major breakpoint region of the *bcl-2* gene (5' GAG TTG CTT TAC GTG GCC TG 3', which binds to the MBR at the 3' UTR of the *bcl-2* gene at bp 2997 to 3016)⁸ and the JHa region of the immunoglobulin heavy chain joining region (5' ACC TGA GGA GAC GGT GAC C 3'), in a microvolume fluorimeter (LightCycler, Idaho Technology, Idaho Falls, ID).¹⁶ Briefly, 50 ng of purified DNA was amplified in a 10- μ l reaction in glass capillary tubes containing 50 mmol/L Tris (pH 8.5), 3.0 mmol/L $MgCl_2$, four deoxynucleotide triphosphates at 200 μ mol/L each, primers at 0.5 μ mol/L, and 0.4 U of *Taq* DNA polymerase (Promega, Madison, WI) with 11 ng/ μ l TaqStart antibody (ClonTech, Palo Alto, CA) per 10- μ l sample. A hot start technique was used in all assays (94°C for 1.5 minutes) followed by 45 cycles of denaturation (94°C for 10 seconds), annealing (68°C for 0 seconds), and extension (74°C for 20 seconds). Each reaction included SYBR Green I (1:30,000 dilution; Molecular Probes, Eugene, OR), which interacts with all dsDNA. In the LightCycler, filtered excitation light (450 to 490 nm) from a blue-light-emitting diode is reflected from a 505-nm epi-illumination dichroic filter and focused on the capillary tip where samples are interrogated by paraxial epi-illumination. A proportion of the excitation light is conducted up the capillary tube by total internal reflection at the glass air interface. Emitted light is similarly conducted down the capillary tube, exits via the tip and is filtered through a 520- to 560-nm interference filter and focused onto silicon photodiodes for detection. Data acquisition is achieved using Labview graphical programming language (National Instruments, Austin, TX) with a 12-bit multifunction input-output card in a 120-MHz Pentium microcomputer (Intel, Santa Clara, CA).¹⁶ Fluorescence signals were obtained once in each cycle by sequential monitoring of fluorescence of each tube for 70

Table 1. Summary of the Results of Conventional and Fluorescence PCR and DNA Sequencing Analyses

Case	Conventional PCR and gel analysis	Fluorescence PCR	EBV PCR	T_m (°C)		G/C content (%)	MBR/JH PCR product size (bp)
				Observed	Calculated		
1	+	+	—	89.8	91.1	60.4	192
2	+	+	—	90	87.4	55.55	207
3	+	+	—	88.2	89	55.2	193
4	+	+	—	89.3	90.3	58.6	184
5	+	+	—	89.5	87.8	52.2	197
6	+	+	—	89.5	91	60.9	174
7	+	+	—	89	88.6	52.7	254
8	+	+	—	89.3	86	46.66	195
9	+	+	—	88.4	86.7	50.2	179
10	+	+	—	88.8	90.7	58.68	213
11	+	+	—	89.1	90.8	59.13	208
12	+	+	—	89.2	89.3	55.5	209
13	+	+	—	89.5	91.8	63.3	161
14	+	+	—	89.6	92	63.5	170
15	+	+	+	88.6	85.3	47	170
16	+	+	—	89.5	91.2	59.5	237
17	+	+	—	88.7	86.8	51.57	158
18	—	—	+		NA	NA	
19	—	—	+		NA	NA	
20	+	+	—	87.7	87.7	56.2	135
21	—	—	—		NA	NA	
22	+	+	—	88.5	90.9	60.22	181
23	—	—	+		NA	NA	
24	—	—	+		NA	NA	
25	—	—	+		NA	NA	
26	—	—	+		NA	NA	
27	—	—	—		NA	NA	
28	—	—	—		NA	NA	
29	—	—	—		NA	NA	
30	—	—	—		NA	NA	

NA, not applicable.

milliseconds at the end of extension. All runs included a negative DNA control (normal human tonsil), positive control (SUDHL-6 cell line DNA), and a control without template. PCR amplification was performed under identical conditions with primers specific for the human β -globin gene to confirm the amplifiability of the sample DNA. The forward primer sequence was 5' GGC TTC CTA GAG ACC AAT CA 3' (GenBank accession U01317, bases 47693 to 47712), and the reverse primer sequence was 5' AAC CAA GAC AGC CAG TTC AC 3' (GenBank accession U01317, bases 47780 to 47799). Multiplex PCR was also performed using both the bcl-2/JH primers and the β -globin primers in the same reaction to demonstrate the specificity of the fluorescence melting characteristics of the different products in the same reaction. After PCR amplification, the PCR products were cooled to 45°C and then slowly heated to 100°C at a rate of 0.2°C per second. For easier visualization of the melting temperatures, fluorescence melting peaks were derived from the initial fluorescence melting curves (F versus T) by plotting the negative derivative of fluorescence over temperature versus temperature ($-dF/dT$ versus T). Fluorescence PCR analysis was also performed using serial dilutions of SUDHL-6 DNA as described above for conventional PCR. The melting temperatures for both the MBR/JH and β -globin PCR products were also calculated using the equation $T_m = 81.5 + A + 0.41 \times (\% \text{ G/C}) - (500/\text{bp})$, where $A = 16.6 \times \log_{10} \{[\text{Salt}]/(1 + 0.7 \times [\text{Salt}])\}$, and $[\text{Salt}] = [\text{Na}^+] + 4[\text{Mg}^{2+}] - \text{dNTP}^{0.5} + [\text{Tris}^+]$ (T_m is

melting temperature, and bp is length of product).¹⁷ Both the calculated and observed T_m values for each sample are presented in Table 1.

Multiplex PCR

Multiplex PCRs were performed using MBR and JH primers as well as the β -globin primers as stated above for fluorescence MBR/JH PCR.

DNA Sequencing and Analysis

DNA sequencing analysis was performed on all 19 cases showing positive fluorescence signals for the MBR/JH product (Table 1). The PCR amplification products for DNA sequencing were purified and concentrated using a Microcon-30 microconcentrator (Amicon, Beverly MA). Template DNA concentrations were determined by absorbance at 260 nm. The purified PCR products were directly sequenced using the same primers as for the MBR/JH PCR reactions on the ABI PRISM 377 with dye terminators (PE Applied Biosystems, Foster City, CA). The identity of the PCR product sequences was confirmed using the BLAST NCBI database.

Dilution Analyses

Dilutional assays were performed using serial dilutions of the MBR/JH-positive SUDHL-6 DNA, starting with 50

ng/ μ l as the initial template concentration. Fluorescence melting curves, relative fluorescence *versus* cycle number plots, and the area under the curves of fluorescence melting peaks were monitored.

Statistical Analysis

The empirically determined and calculated T_m values for each of the 19 positive samples were analyzed using the paired two-tailed Student's *t*-test, which indicated that there was no statistically significant difference between both sets of data ($P = 0.8164$).

Results

The results of the PCR and DNA sequencing analyses are summarized in Table 1.

β -Globin PCR

All 30 cases analyzed yielded the expected 107-bp β -globin product by conventional PCR as well as the characteristic decline in fluorescence (Figure 1A) and fluorescence melting peak at 81.2°C by fluorescence PCR indicating adequate DNA integrity for PCR (Figure 1b). The expected T_m of the β -globin product was calculated to be 80.8°C using the equation described above.

MBR/JH PCR

All 19 cases with MBR/JH rearrangements determined by conventional PCR and gel electrophoresis showed an abrupt decline in fluorescence signal (F) at $88.85 \pm 1.15^\circ\text{C}$ on the F *versus* T curves (Figure 2A). A corresponding melting peak was evident at $88.85 \pm 1.15^\circ\text{C}$ on the $-dF/dT$ *versus* T graphs in all cases with the MBR/JH translocation product (Figure 2B illustrates a case with T_m of 90°C). All positive and negative controls gave appropriate results. The 11 cases that were negative for the translocation by conventional PCR were also negative by fluorescence PCR. These results indicate a perfect correlation between the findings on conventional PCR and gel electrophoresis and fluorescence PCR.

Multiplex MBR/JH and β -Globin PCR

Fluorescence melting curve analysis of multiplex PCR using MBR and JH primers as well as β -globin primers in the same reaction revealed sharp declines in fluorescence and the appropriate fluorescence melting peaks at 81.2°C and 90°C , corresponding to the specific amplification products of β -globin and MBR/JH, respectively (Figure 3).

Dilution Analyses

Dilutional assays were performed using serial dilutions of the MBR/JH-positive SUDHL-6 DNA; starting with 50 ng/

Figure 1. A: Fluorescence melting curve for β -globin. Melting curves were acquired after 45 cycles of amplification with primers to the human β -globin gene. After PCR amplification, the PCR products were cooled to 45°C and then heated from 45°C to 100°C at a rate of 0.2°C per second. **Curve A**, melting curve obtained from a template-free control (distilled H_2O); **curve B**, melting curve obtained from amplification of the specific 107-bp β -globin product from human placental DNA. A sharp decline in fluorescence characteristic of the β -globin product is evident at 81.2°C . **B**: Fluorescence melting peak for β -globin. Fluorescence melting peaks were obtained by plotting the negative derivative of fluorescence over temperature *versus* temperature ($-dF/dT$ *versus* T). **Curve A**, fluorescence profile of the H_2O control; **curve B**, characteristic melting peak (81.2°C) obtained for the β -globin product.

10- μ l reaction as the initial template concentration. Fluorescence melting curves, relative fluorescence *versus* cycle number plots and the area under the curves of fluorescence melting peaks were analyzed. Examination of the fluorescence profiles derived from five amplification reactions consisting of sequential decreases in initial template concentration (ie, 50, 5, 0.5, 0.1, and 0.05 ng, all in 10- μ l amplification reactions) revealed that the cycle number at which logarithmic amplification was noted was inversely related to the starting copy number of template (data not shown). At low initial template copy numbers, quantitation is difficult because of the formation of and inability to distinguish undesired products. However, melting peak analysis permitted the discrimination of the desired MBR/JH product from primer dimers at initial template concentrations as low as 0.05 ng in a 10- μ l reaction (Figure 4A).

By contrast, definitive detection of MBR/JH product by conventional PCR and agarose gel electrophoresis was limited to a concentration of 5 ng/10- μ l reaction (Figure 4B). This result indicates that fluorescence melting peak

Figure 2. A: Fluorescence melting curve for MBR/JH translocation product. Melting curves were acquired under identical conditions as for the β -globin PCR above. **Curve A**, melting curve obtained from a template-free control (distilled H_2O); **curve B**, melting curve obtained from the amplification product from human placental DNA; **curve C**, melting curve obtained from the 256-bp MBR/JH product using the SUDHL-6 cell line. A sharp decline in fluorescence characteristic of the MBR/JH product is evident at 90.5°C. **B**: Fluorescence melting peak for MBR/JH translocation product. Fluorescence melting peaks were obtained as stated for β -globin above. **Curve A**, fluorescence profile of the template-free control (H_2O) (note the broad nonspecific melting peak from the primer dimer); **curve B**, melting curve obtained from amplification of human placental DNA; **curve C**, characteristic melting peak (90.5°C) obtained for the 256-bp MBR/JH (SUDHL-6) translocation product. **C**: Fluorescence melting peaks for different MBR/JH products. Fluorescent melting peaks of three different MBR/JH products showing slightly different melting temperatures (T_m): **curve B**, $T_m = 89.3^\circ C$ (184 bp); **curve C**, $T_m = 90^\circ C$ (192 bp); **curve D**, $T_m = 90.5^\circ C$ (207 bp). The fluorescence profile of the negative control (human placental DNA) is represented by **curve A** and shows only a primer dimer peak.

analysis can be more sensitive than conventional methods by up to two orders of magnitude.

EBV-PCR

EBV DNA was demonstrable by PCR analysis in 7 of the 30 DNA samples examined (Table 1). Six of the EBV-positive samples were obtained from hyperplastic lymphoid tissues and were negative for MBR/JH PCR by both conventional and fluorescence PCR, supporting the specificity of the fluorescence PCR method. One case (case 15) was positive for junctional MBR/JH sequences and contained EBV viral genome but yielded only a fluorescence curve with T_m characteristic of the MBR/JH product T_m (88.6°C; Table 1).

Sequence Analysis

Bidirectional DNA sequencing confirmed the presence of the MBR/JH translocation in the 19 cases detected by fluorescence melting curve analysis.

Discussion

Integrating nucleic acid amplification with PCR product analysis obviates the necessity for electrophoresis. In general, fluorescence monitoring of PCR may employ a nonspecific dsDNA-binding dye or sequence-specific fluorescently labeled probes.¹¹ These fluorescently labeled probes are typically expensive and require some sophistication in their design for optimal monitoring of fluorescence resonance energy transfer. On the other hand, dsDNA-binding dyes, such as ethidium bromide and SYBR Green I, are relatively inexpensive and easy to use. *In vitro* nucleic acid amplification with simultaneous quantitative monitoring of PCR product formation can be achieved by incorporation of dsDNA-binding dyes (eg, ethidium bromide,¹⁸ YO-PRO 1,¹⁹ and SYBR Green I¹¹) into PCR. In this regard, we have used SYBR Green I because it exhibits an excitation maximum (497 nm) similar to fluorescein and appears to produce a stronger signal with DNA than does ethidium bromide. Once-per-cycle acquisition of fluorescence signal during the annealing or annealing/extension phases of each thermal cycle reveals an increase in fluorescence signal related to progressive PCR product accumulation. Continuous monitoring of amplification reveals that increased temperature leads to dsDNA denaturation and predominance of single-stranded species and thus less target for binding by the dsDNA-binding dyes. Hence, when dsDNA is denatured, the binding of the dsDNA dye is diminished, leading to a corresponding decrease in fluorescence. A specific PCR product may thus be identified by its specific fluorescence melting curve, because its melting temperature (T_m) is dependent on its GC content, length, and sequence.

In this study, we demonstrate the feasibility of using DNA melting curves for the detection of MBR/JH translocations without employing fluorescently labeled probes.

Although SYBR Green I exhibits nonspecific binding to dsDNA species other than the specific MBR/JH product, the fluorescence signal obtained from primer dimers is of a substantially lower melting point and is thus readily distinguishable from that of the desired PCR product (Figures 2B and 4A). The relationship between T_m , GC content, and DNA length is evidenced by the lower T_m ($\sim 81^\circ\text{C}$) of the β -globin PCR product, which has a shorter length (107 bp) and a lower GC content (40.4%) than the MBR/JH product with a higher T_m ($\sim 90^\circ\text{C}$), greater length (mean, 190.4 bp), and higher GC content (mean, 56.18%).

Similar to the detection of MBR/JH by the migration of the PCR products within an expected size range, fluorescence PCR yields products over a temperature range ($88.85 \pm 1.15^\circ\text{C}$). This also reflects the fact that different products result from the juxtaposition of different MBR breakpoints to JH region sequences (Figure 2C). Unlike agarose gel electrophoresis, however, fluorescence melting curve analysis can sometimes discriminate between products of almost identical size but with different GC content (Table 1, samples 1 and 3).

Inadvertent amplification of unintended sequences is a potential pitfall of PCR. Of relevance to MBR/JH PCR, a commonly used MBR primer (bases 2992 to 3011 in the *bcl-2* gene) contains nine sequential bases that are also present in the EBV genome at its 3' end, with 14 oligonucleotides matching in total.¹⁵ In addition, the JH primer also contains nine sequential complementary bases to the EBV genome with 14 matching bases overall. Both primers show greatest sequence homology with the EBV genome at their 3' ends, which is most crucial for specific primer annealing and the subsequent extension step.

To determine the specificity of the fluorescence PCR assay, we examined six cases of known EBV-positive, t(14;18)-negative lymphoproliferative disorders using a similar MBR primer (bases 2997 to 3016 in the *bcl-2* gene, with a total of 15 bases with homology to the EBV genome) and a consensus JH primer. In all cases, the

Figure 3. Fluorescence melting peaks for MBR/JH and β -globin multiplex PCR. Fluorescence melting peaks were obtained from a multiplex PCR using both MBR/JH and β -globin primers. **Curve A**, fluorescence profile of the template-free control (H_2O); **curve B**, melting peak for the β -globin product only (81.2°C); **curve C**, melting peak for the MBR/JH product only (90.5°C); **curve D**, melting peak for the multiplexed β -globin and MBR/JH PCR, yielding both the β -globin product (81.2°C) peak and the MBR/JH (90.5°C) peak.

Figure 4. A: Dilutional analysis by fluorescence melting peak analysis. Fluorescence melting curves are shown for serial dilutions of SUDHL-6 DNA using an initial template concentration of 50 ng/10 μl of PCR reaction. MBR/JH product melting at 90.5°C is evident at dilutions as low as 1:1000. **B:** Dilutional analysis by conventional PCR, agarose gel electrophoresis, and UV transillumination. A 10- μl volume of PCR product was loaded in each lane. The initial template concentration was 50 ng/10- μl reaction. **Lane 1**, DNA ladder with band sizes of 1000, 700, 525/500, 400, 300, 200, and 100 bp from origin; **lane 2**, MBR/JH-positive control (SUDHL-6) at 50 ng/10- μl reaction; **lane 3**, 1:2 dilution of initial template concentration; **lane 4**, 1:5 dilution of initial template concentration; **lane 5**, 1:10 dilution of initial template concentration; **lane 6**, 1:50 dilution of the initial template concentration. MBR/JH product is detectable only as low as a 1:10 dilution of the initial template (lane 5).

characteristic melting peak at $88.85 \pm 1.15^\circ\text{C}$ was absent, indicating specificity of the assay for MBR/JH product. We also examined a case of MBR/JH-positive FCL that also contained EBV genome (case 15). Fluorescence PCR analysis revealed a PCR product with T_m of 88.6°C , and DNA sequencing analysis confirmed that the product contained only MBR/JH junctional sequences, further verifying assay specificity (Table 1). Our fluorescence PCR method features a high annealing temperature 68°C for 0 seconds, which discourages any nonspecific hybridization between the primers and an irrelevant template. In the unlikely event that unintended sequences are amplified at such a high annealing temperature, the melting characteristics of the resulting product would be different from that obtained for the MBR/JH product and thus easily distinguished. Multiplex PCR assays using both MBR/JH and β -globin primers also demonstrate the specificity of fluorescence melting peak analysis (Figure 3). Comparison of conventional PCR followed by gel electrophoresis with fluorescence PCR analysis reveals that

melting curve analysis identifies MBR/JH product at much lower initial template concentrations than with ethidium-bromide-stained gels (Figure 4, a and b). When compared with 5' exonuclease-based assays using sequence-specific probes, the dynamic range of fluorescence monitoring using DNA binding dyes is limited by nonspecific detection of undesired products at very low template concentration and after excessive PCR cycling.^{11,20}

Whereas bcl-2/JH fusion sequences have been detected in the peripheral blood of healthy individuals²¹ and in hyperplastic lymphoid tissues,²² none of the cases of reactive lymphoid hyperplasia examined in our study yielded a positive fluorescence signal for MBR/JH junctional sequences. This may be attributed to the comparatively low initial template DNA concentration (50 ng/reaction) used in our assays. Nevertheless, we recommend correlation of genetic studies with histopathological and clinical findings before primary diagnosis of FCL is rendered. In cases in which recurrence of the primary neoplasm is suspected on account of detection of MBR/JH sequences, it is advisable to examine the clonal relatedness of sequential lymphomas by DNA sequencing analysis.

In conclusion, we have demonstrated that fluorescence melting peak analysis is a rapid, inexpensive, and accurate method for the detection of MBR/JH translocations. This method permits the simultaneous amplification, detection, and quantification of the specific PCR product over a broad range of initial template concentration. The simplicity and rapidity of this method render it very attractive for routine use in other spheres of molecular pathology.

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