Real-Time t(14;18)(q32;q21) PCR Assay Combined with High-Resolution Capillary Electrophoresis: A Novel and Rapid Approach that Allows Accurate Quantitation and Size Determination of *bcl-2/JH* Fusion Sequences

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Follicular lymphoma is characterized by the presence of the t(14;18)(q32;q21) chromosomal translocation that juxtaposes the *bcl*-2 gene at 18q21 with the immunoglobulin heavy chain (*Ig*H) locus at 14q32. We have previously shown that accurate quantitation of t(14;18)-carrying cells in follicular lymphoma patients can be achieved by non–gel-based real-time TaqMan polymerase chain reaction (PCR; Applied Biosystems, Foster City, CA). Since our report, several studies have demonstrated that real-time PCR is highly sensitive and a reliable tool for evaluating treatment effectiveness and for following minimal residual disease in follicular lymphoma patients. Unfortunately, currently available real-time PCR methods do not determine the size of the amplification product, which is useful for excluding contamination and is commonly used as presumptive evidence of clonal identity or disparity when multiple samples from the same patient are analyzed. We describe a modified real-time PCR assay that rapidly allows accurate quantitation and precise determination of the size of the t(14;18) fusion sequence without the need for gel electrophoresis. In this assay, a consensus immunoglobulin heavy chain–joining region gene (*JH*) primer labeled at its 5' end with the fluorescent dye NED (Applied Biosystems) is included in the real-time PCR assay and thus is incorporated into the *bcl*-2/*JH* fusion product. The JH-NED primer did not interfere with the TaqMan probe fluorescent signal or target detection and allowed subsequent amplicon size determination by semiautomated high-resolution capillary electrophoresis.

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Real-time polymerase chain reaction (PCR) for detection and quantitation of *bcl*-2/*JH* fusion sequences characteristic of t(14;18) (q32;q21) is being used increasingly to monitor tumor burden and the effect of therapy in patients with follicular lymphoma (FL; 1–5). Real-time PCR methods enable t(14;18) detection without the need to open amplification tubes and are target specific, highly sensitive, and reproducible. Thus, real-time PCR methods have several advantages over conventional PCR techniques that require gel electrophoresis–based amplicon detection and quantitation techniques.

Currently available real-time PCR methods to detect the t(14;18) do not allow amplicon size determination. This is a major drawback for a few reasons. First, one cannot easily exclude contamination. Second, size of PCR products from multiple samples (either simultaneous or sequential) is a valuable means of determining clonal relatedness, as the size of *bcl*-2/*JH* fusion sequences generally vary from clone to clone (6–8). Undoubtedly, sequencing of the fusion sequences is the most accepted method for determining clonal relatedness, but sequencing methods can be tedious and time consuming. Third, as t(14;18)-positive cells are detected in normal healthy individuals (9–12), the ability to quantitate low-level tumor load after therapy may be compromised by background *bcl*-2/*JH*-positive cells unrelated to the tumor clone.

Although these drawbacks can be addressed using conventional gel electrophoresis, this approach has a limited dynamic range and does not provide accurate evaluation of amplicon size. In addition, this approach defeats the high-throughput automation capability of quantitative real-time TaqMan

(Applied Biosystems, Foster City, CA) technology. Hence, we were interested in developing a PCR assay that does not compromise the real-time detection/quantitation of t(14;18) but allows amplicon size determination rapidly and precisely without the need for conventional gel electrophoresis.

We describe here a novel modification of our real-time t(14;18) PCR assay that combines TaqMan technology with fluorescent-based high-resolution capillary electrophoresis (CE) and GeneScan (GS) DNA fragment analysis (Applied Biosystems) for accurate quantitation and size determination of the *bcl*-2/*JH* fusion sequences. CE is semiautomated and permits distinction of PCR fragments differing in size by one base (13). In addition, the resulting electropherogram that shows the mobility and the molecular size of the *bcl*-2/*JH* fusion sequence can be stored in digital form, allowing easy export to analysis software or exchange with other laboratories for comparative analysis. This novel real-time t(14;18) PCR-CE assay is sensitive, quantitative, and allows high throughput analysis.

MATERIALS AND METHODS

We selected 24 samples from 21 patients with follicular lymphoma (FL) from the files of the Department of Hematopathology, University of Texas–M.D. Anderson Cancer Center (UTMDACC). All cases were diagnosed as FL according to the criteria of the World Health Organization (14). Three cell lines known to carry the t(14;18), involving either the *bcl*-2 major breakpoint region (mbr) or minor cluster region (mcr), were included in this study (gifts from Dr. Richard Ford).

High molecular weight DNA was isolated from cell lines, 20 bone marrow and 4 peripheral blood specimens, in accordance with standard proteinase K digestion and organic extraction procedures. All cell lines were previously confirmed to have t(14;18) by conventional PCR and sequence analysis. DNA obtained from HL60 cell line was used as a negative control.

Real-Time t(14;18)(q32;q21) PCR

Real-time assays were performed using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Each sample was amplified in duplicate using a JH consensus primer in combination with mbr- or mcr-specific primers and 6-carboxyfluorescein (6-FAM) labeled TaqMan probes (Table 1). For the size analysis of *bcl*-2/*JH* fusion sequences by CE, real-time PCR was performed with the JH consensus primer labeled with NED fluorescent dye at its 5' end (JH-NED). The PCR was performed in a final volume of 25 μL containing 1 μg of genomic DNA, 1× TaqMan buffer, 5.0 mmol/L $MgCl_2$, 0.2 μmol/L of each primer, 2.5 μmol/L of TaqMan probe, 480 μmol/L dUTP, 240 μmol/L, dATP, 240 μmol/L dCTP, 240 μmol/L dGTP, 0.25 U of AmpErase uracil-*N*-glycosylase (UNG), and 0.625 U of AmpliTaq Gold polymerase. To prevent carryover contamination, UNG was used in the PCR mix.

Each sample was subjected to 40 cycles of PCR. The PCR thermal-cycling conditions included UNG activation at 50° C for 2 minutes, followed by TaqGold activation and DNA denaturation step for 12 minutes at 95° C. Each cycle consisted of 95° C denaturation for 30 seconds, 58° C annealing for 20 seconds, and 72° C extension for 45 seconds. The last cycle was followed by a 10-minute elongation step at 72° C.

Amplification of a 93-bp sequence from the *cyclophilin* (15) gene was performed simultaneously in all cases to confirm the quality of extracted DNA and to normalize t(14;18) values.

TaqMan Data Analysis

The fluorescence emission data for each sample was analyzed immediately after PCR using Sequence Detection Software (SDS version 1.7, Applied Biosystems) as described previously (2). The threshold cycle (Ct) values of each sample and cell line were exported into Microsoft Excel software for further analysis. The Ct represents the PCR cycle number at which fluorescence signal is increased above an arbitrary threshold.

TABLE 1. Primers and Probes Used in Real-Time Polymerase Chain Reaction and Capillary Electrophoresis–GeneScan DNA Fragment Analysis

Substance Primer	Sequences
bcl-2 mbr-F	5′-GCT TTA CGT GGC CTG TTT CA-3′
bcl-2 mcr-F	5′-CCT GGC TTC CTT CCC TCT GT-3′
JH-R	5′-NED -ACC TGA GGA GAC GGT GAC C-3′
cy-F	5′-TGA GAC AGC AGA TAG AGC CAA GC-3′
cy-R	5′-TCC CTG CCA ATT TGA CAT CTT C-3′
Probe	
bcl-2 mbr-P	5′-FAM-AGG GCT CTG GGT GGG TCT GTG TTG-TAMRA-3′
bcl-2 mcr-P	5′-FAM-TCT CTG IGG AGG AGT GGA AAG GAA GG-TAMRA-3′
cy-P	5′-VIC -AGC ACC AAT ATT CAG TAC ACA GCT TAA AGC TAT-TAMRA-3′

mbr, major breakpoint region; mcr, minor cluster region; F, forward; R, reverse; P, probe; *cy*, cyclophilin; JH, joining region of immunoglobulin heavy chain gene.

Cell Line Standards for Quantitation of t(14;18)

Serially diluted DNA from cell lines known to carry t(14;18) translocation were used to generate standard curves. Briefly, 1000 ng of DNA from each cell line, equivalent to approximately 120,000 to 160,000 cells, was serially diluted into 10 mM Tris buffer, pH 8.0, to achieve 10^{-1} to 10^{-5} fold dilutions and then subjected to real-time PCR. Standard curves were generated by plotting Ct *versus* the amount of target DNA in each dilution. The standard curves for *bcl*-2-mbr/*JH and bcl*-2-mcr/*JH* were similar whether cell line DNA was diluted into Tris buffer or into HL60 cell line DNA (data not shown). Hence, the DNA diluted in Tris buffer was used to generate standard curves to calculate the quantities of *bcl2-/JH* fusion sequences and *cyclophilin* in a test sample.

GeneScan Analysis

Immediately after real-time PCR including JH-NED, 0.5 μL of each amplification product was subjected to CE in an ABI Prism 310 Genetic Analyzer (Applied Biosystems) with POP4 polymer. The 310 Genetic Analyzer is a laser-based fluorescence detection system and automatically introduces the samples labeled with fluorescent dyes into a polymer-filled capillary for electrophoresis. We used GenoTYPE-ROX 50-500 DNA ladder (Life Technologies, Gaithersburg, MD) as internal size standards. The size of each fragment is calculated with GS software using the Local Southern sizing option (Applied Biosystems). The internal size standards, which are fluorescently labeled, were included in each sample to ensure correct base calling and alignment of the peak patterns.

RESULTS

Sensitivity of the Modified Real Time PCR Assay for t(14;18)

We tested the effect of using a JH-NED primer on the sensitivity of the TaqMan PCR assays for t(14;18) by analyzing serially diluted cell line DNA in the presence of JH-NED or unlabeled JH primers. As shown in Figure 1, the Ct values over a 5-log dynamic range of starting target copy number were similar whether JH-NED or unlabeled JH primer was used. This indicates that NED did not interfere with the real-time detection of *bcl-2* mbr/*JH* and *bcl-2* mcr/*JH* fusion sequences.

We also tested two t(14;18)-positive cell lines with *bcl*-2 mbr/*JH* fusion sequences that differed in size by 125 bases to determine whether the efficiency of our modified PCR assay was influenced by amplicon size. Standard curves generated using these two cell lines showed good correlation ($r = 0.989$), indicating that the efficiency and the sensitivity of the modified real-time t(14;18) PCR assay is not altered by variation in amplicon size (data not shown).

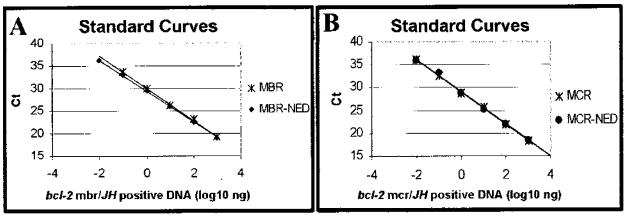

FIGURE 1. Standard curves showing the initial DNA quantity *versus* threshold cycle (Ct). The standard curves were generated by real-time PCR assays using serial dilutions of (14;18)-positive cell line DNA and either unlabeled JH primer (◇) or JH primer labeled with the fluorescent dye NED (*).

Sensitivity of CE and GS for t(14;18)

After real-time PCR using JH-NED primer and cell line DNA (positive controls), the products were analyzed by CE and GS (Fig. 2). A single peak of either 119 or 220 bases was detected depending on whether *bcl*-2 mcr/*JH*- or *bcl*-2 mbr/*JH*-positive cell line DNA was amplified, respectively. Sequence analysis of the fusion sequences confirmed the accuracy of the sizes determined by GS. No signal was observed in the negative control (HL60 cell line). We then analyzed the fluorescent-labeled PCR products derived from serial dilution studies. In these studies, genomic DNA from HL60 cell line was used as t(14;18)-negative DNA to dilute t(14;18)-positive cell line DNA. As observed with the real-time PCR methods (Fig. 2A–B), a fluorescence signal was detected by GS (Fig. 2C–D) at all dilutions including 10^{-5}, which is equivalent to approximately one translocation-bearing cell in the background of 120,000 normal cells. The sensitivity of CE-GS for t(14;18) at this low tumor burden, even though only 0.5 μL of the PCR product was analyzed, validates the utility of this assay as a tool for monitoring minimal residual disease after therapy in FL patients.

Figure 3 illustrates the simplicity and utility of this assay for analyzing multiple patient samples simultaneously. Samples from nine patients with FL were subjected to real-time PCR, and the products were analyzed subsequently by CE and GS. Real-time PCR amplification plots for t(14;18) and *cyclophilin* (Fig. 3A–B) demonstrated that each sample had detectable t(14;18) and that tumor burden levels varied among the patients. GS further detected a unique size amplicon for each sample (Fig. 3C), confirming that fluorescent signal detected by real-time PCR was due to patient-specific clone. We analyzed samples from 13 additional FL patients by this assay, and for each sample, we correlated positive fluorescence signal detected by

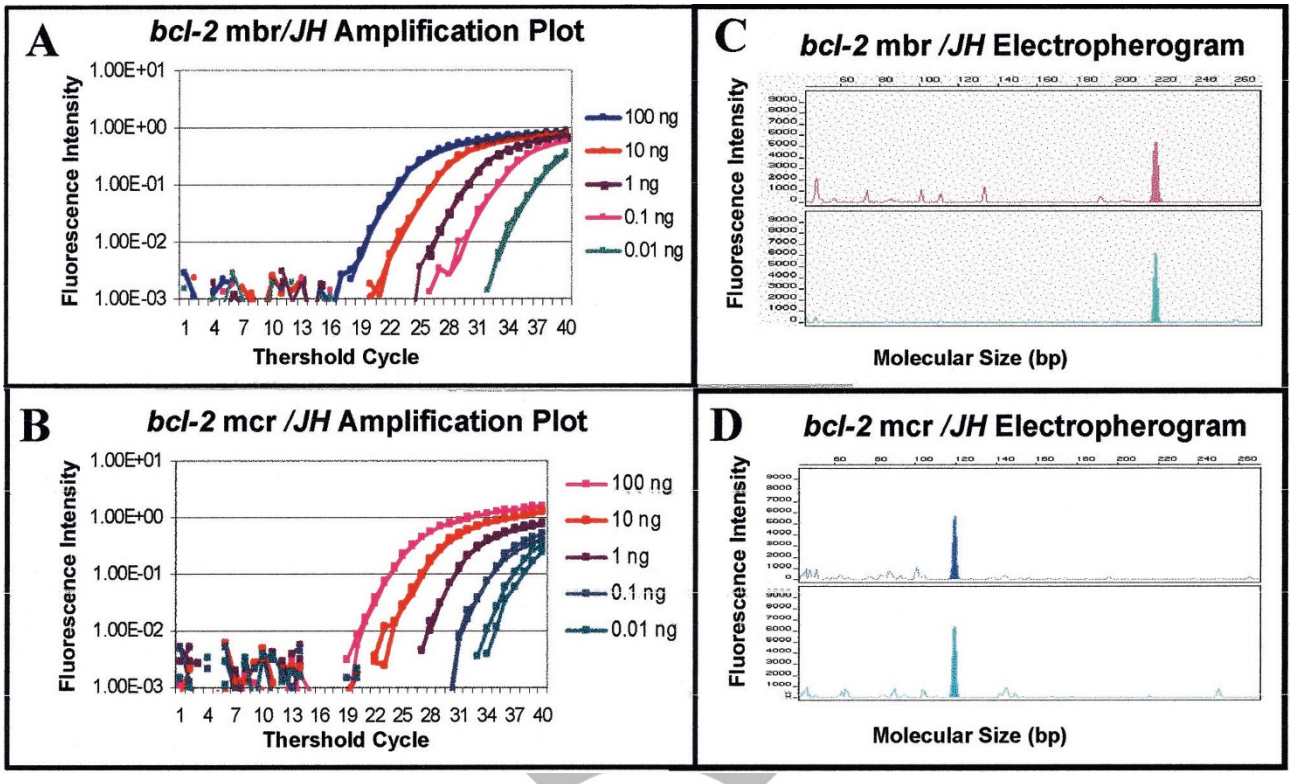

FIGURE 2. Detection sensitivities of modified real-time t(14;18) PCR assays. Left panels show amplification plots generated using 10-fold dilutions of *bcl*-2 mbr/*JH*- (**A**) and *bcl*-2 mcr/*JH*- (**B**) positive cell line DNA. Electropherograms show the mobility and molecular size of NED-labeled PCR products generated by the modified real-time PCR for *bcl-2* mbr/*JH* (**C**) and *bcl-2* mcr/*JH* (**D**). Upper and lower panels of each electropherogram correspond to products generated by real-time PCR using 0.1 ng and 0.01 ng of *bcl*-2 mbr/*JH*- (**A**) and *bcl*-2 mcr/*JH*- (**B**) positive DNA, respectively.

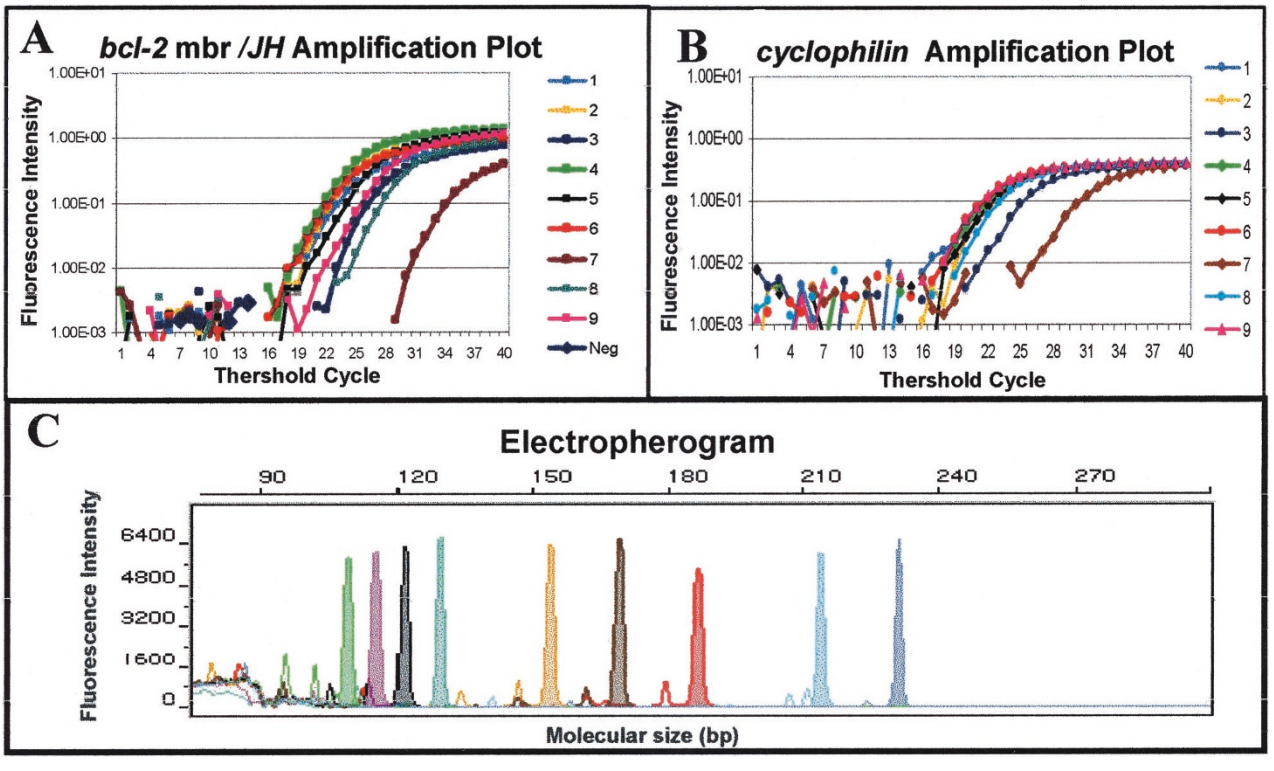

FIGURE 3. *bcl*-2 mbr/*JH* (**A**) and *cyclophilin* (**B**) amplification plots and *bcl*-2 mbr/*JH* electropherograms (**C**) of nine different samples derived from FL patients showing the amount of *bcl*-2/*IgH* and *cyclophilin* and the specific amplicon size in each case. Color is matched for each patient in the amplification plots and corresponding electropherogram.

TABLE 2. Summary of Real-Time Polymerase Chain Reaction and GeneScan Assay Results

Sample No.	Patient No.	Sample Location	*bcl*-2 mbr/*JH* (Ct)	*bcl*-2 mbr/*JH* (ng)	*cy* (Ct)	*cy* (ng)	*bcl*-2-*JH*/*cy*	Size (bp)
1	1	BM	19.64	651.6	19.01	2436.5	2.7E−01	214
2	2	BM	19.53	697.9	19.50	1726.6	4.0E−01	154
3	3	BM	23.13	59.4	22.48	204.1	2.9E−01	231
4	4	BM	18.62	1309.0	19.08	2291.5	5.7E−01	110
5	5	BM	20.19	451.1	19.45	1754.9	2.6E−01	122
6	6	BM	19.09	947.2	18.89	2625.3	3.6E−01	187
7	7	BM	30.41	0.41	27.42	6.1	6.7E−02	189
8	8	BM	25.86	19.1	21.09	936.3	2.0E−02	130
9	9	PB	23.07	105.0	19.55	2566.0	4.1E−02	185
10	10	PB	28.81	2.8	19.65	2389.5	1.2E−03	159
11	11	BM	35.24	0.1	19.66	2380.4	2.0E−05	175
12	12a	PB	27.86	5.1	19.51	2622.0	2.0E−03	179
13	12b	BM	27.22	7.6	19.86	2154.5	3.5E−03	179
14	12c	BM	27.90	4.9	19.84	2152.5	2.3E−03	179
15	13	BM	38.10	0.01	19.84	2152.5	4.0E−06	81
16	14	BM	20.77	522.0	23.88	369.0	1.4E+00	170
17	15	BM	19.70	948.0	20.52	1870.0	5.1E−01	144
18	16	BM	18.78	1560.0	22.12	574.0	2.7E+00	138
19	17	BM	22.10	317.6	19.65	2389.5	1.3E−01	229
20	18	BM	35.03	0.05	31.20	0.4	1.2E−01	223
21	19a	PB	30.15	1.2	19.89	2043.9	6.0E−04	161
22	19b	BM	29.48	1.8	19.67	2358.1	8.0E−04	161
23	20	BM	32.44	0.3	20.07	1811.2	2.0E−04	139
24	21	BM	22.34	216.0	20.75	1620.0	1.3E−01	116

mbr, major breakpoint region; mcr, minor cluster region; *cy*, cyclophilin; Ct, threshold cycle; bp, base pairs; BM, bone marrow; PB, peripheral blood.

TaqMan assay with an amplicon of unique size by GS (Table 2). The size of the *bcl*-2/*JH* amplification product ranged from 81 to 231 bp among the 21 patient samples. Two patients (Patients 12 and 19) had sequential samples in which an identically sized *bcl*-2/*JH* fusion sequence was amplified. It is interesting to note that though we used the same amount of DNA in all assays, some samples showed very low levels of cyclophilin, indicating poor quality of the DNA or the presence of inhibitors in the samples.

DISCUSSION

Although the majority of patients with indolent FL achieve clinical remission after induction therapy, they continue to have low-level disease that eventually leads to clinical relapse. Several studies have recently demonstrated that achievement of molecular complete remission is a desirable goal of new therapies because patients with molecular complete remission have longer disease-free status (16–18). The clinical importance of achieving molecular response has been demonstrated in FL patients treated with several regimens, including rituximab (18, 19) and stem cell therapy (3). Moreover, quantitative results may provide more prognostic information in the assessment of minimal residual disease detected by PCR analysis of peripheral blood, bone marrow, and stem-cell preparations used for transplantation after high-dose chemotherapy.

Since our original report on the application of real-time PCR for t(14:18), several investigators have demonstrated the utility and reliability of this technique in quantitating tumor burden in FL patient samples (2–5, 20, 21). For example, Hirt and Dolken (3) have demonstrated that quantitative detection of circulating t(14;18) by real-time PCR in follow-up samples of FL patients after autologous bone marrow transplantation predicts clinical course of the disease. Similarly, Ladetto *et al.* (5) have shown that tumor burden in stem cell harvests detected by real-time PCR for t(14;18) can predict the effectiveness of therapeutic intervention in FL patients. These studies demonstrate that real-time PCR is a reliable tool that can be used for monitoring minimal residual disease and in the evaluation of treatment effectiveness in FL patients.

Unfortunately, quantitative real-time PCR methods to assess the t(14;18) do not allow accurate size determination of the products. This is a drawback of this approach as one cannot easily exclude contamination or compare product size in multiple patient samples. Hence, we were interested in developing an assay that does not compromise the real-time PCR detection and quantitation of t(14;18) but permits amplicon size determination rapidly and precisely without the need for conventional gel electrophoresis. Our strategy to accomplish this goal was to label PCR products during real-time PCR by incorporation of a fluorescent dye–labeled primer and then to separate the labeled products by CE-GS. This approach resulted in an assay that did not interfere with TaqMan detection of the target and allowed a precise size determination of each *bcl*-2/JH fusion sequence.

Our original TaqMan t(14;18) PCR assay reported previously used a probe labeled with 6-FAM at the 5'

end and 6-carboxy-tetramethyl rhodamine (TAMRA) at the 3' end. Via fluorescence resonance energy transfer, the fluorescence signal of 6-FAM (donor) is quenched by TAMRA (acceptor) until the probe is hydrolyzed by the exonuclease activity of Taq polymerase. The assay buffer also includes 6-carboxy-X-rhodamine (ROX) as a passive internal reference dye to correct for the fluorescence fluctuations resulting from changes in volume or concentration due to pipetting errors. Thus, our original TaqMan t(14:18) PCR assay includes three fluorophores for target detection. Any strategy that adds another fluorophore to this system requires careful consideration of (1) spectral overlap of the new fluorophore with the TaqMan fluorescent dyes and (2) the possible quenching effect of the new dye on the fluorescence signal of the TaqMan reporter dye. We chose NED for this modified assay because 6-FAM is already used in our assay to label *bcl-2* specific TaqMan probes, ROX is in the buffer, and other dyes have significant spectral overlap with 6-FAM. The modified real-time PCR that included the JH-NED primer did not quench the fluorescent signal of the reporter dye 6-FAM and detected *bcl-2 /JH* fusion sequences in all cases previously shown to have the t(14;18) by our original real-time PCR assay as well as by conventional PCR.

In conclusion, the coupling of real-time t(14;18) PCR with CE that we describe in this report does not require gel-based detection methods, is sensitive, and provides an easy alternative to DNA sequencing for assessing clonal relatedness, which is useful when real-time PCR methods are used to evaluate minimal residual disease. The assay also minimizes the risk of misinterpreting false-positive results that may arise from contamination. In this assay, during real-time PCR, the fluorescent dye NED is incorporated into the *bcl-2/JH* fusion products. This dye did not interfere with target detection but allowed subsequent separation of *bcl-2/JH* fusion sequences by semiautomated CE.

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