Rapid and Accurate Detection of Monoclonal Immunoglobulin Heavy Chain Gene Rearrangement by DNA Melting Curve Analysis in the LightCycler System

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The detection of immunoglobulin heavy chain gene rearrangement (IgH-R) is a standard tool for distinguishing polyclonal from monoclonal B-cell populations. Current DNA-based polymerase chain reactions (PCR) strategies can diagnose monoclonal IgH-R either by measuring the length of the amplicon or by detecting gel mobility variations owing to sequence-dependent conformational changes. However, amplification and analysis remain sequential operations usually requiring manual transfer. We have developed a novel PCR strategy for detecting monoclonal IgH-R that monitors fluorescence of the specific double-stranded DNA binding dye SYBR Green I during melting curve analysis using the LightCycler System. We compared polyacrylamide gel electrophoresis (PAGE) versus melting curve analysis in 130 clinical DNA samples from formalin-fixed, paraffin-embedded (FFPE) tissues (mostly skin biopsies) of 128 patients. The identical FR3 primers were used to amplify the IgH variable region for both analytic techniques. We detected IgH-R in 24 DNA samples from FFPE tissue of 22 patients. Melting curve analysis, compared to PAGE, revealed no false negative and no false positive results, yielding both sensitivity and specificity equal to 100%. We also compared Southern blot analysis versus melting curve analysis in 23 clinical DNA samples from fresh-frozen lymph nodes of 23 patients. We detected IgH-R by melting curve analysis in 7 DNA samples from fresh-frozen lymph nodes. Melting curve analysis, compared to Southern blot analysis, revealed sensitivity equal to 58.3% (7 of 12) and specificity equal to 100% (11 of 11). We conclude that continuous fluorescence monitoring of PCR products with DNA melting curve analysis can rapidly and reproducibly distinguish polyclonal from monoclonal B-cell populations. (*J Mol Diagn* 2002, 4:216–222)

Detecting antigen receptor gene rearrangements has become a standard laboratory test for distinguishing polyclonal from monoclonal B-cell populations. Both Southern blot and polymerase chain reaction (PCR) techniques work well.^{1,2} In recent years, PCR has become the method of choice because of its more rapid turn-around time, lower cost, and greater technical robustness in analyzing DNA extracted from formalin-fixed, paraffin-embedded (FFPE) tissues.^{1,2} Current DNA-based PCR strategies for detecting monoclonal immunoglobulin heavy chain (IgH) gene rearrangement (IgH-R) rely either on measuring the length of the amplicon^{3–5} or on detecting gel mobility variations owing to sequence-dependent conformational changes.^{6–8}

The melting temperature (T_m) of duplex DNA reflects fundamental material properties that depend on length, sequence, G:C content, and Watson-Crick base pairing.^{9,10} DNA duplex melting has been used to assay DNA mutations,¹¹ single nucleotide polymorphism,⁷ and variations in DNA length and sequence.⁸ We hypothesized that a DNA-based PCR strategy for detecting monoclonal IgH-R, based on differences in internal melting domains corresponding to the IgH hypervariable region, could be developed using melting curve analysis. In this report, we demonstrate that amplicons derived from the IgH variable region yield highly reproducible DNA melting curves that can be used to rapidly and reliably diagnose monoclonal IgH-R.

Materials and Methods

Patient Samples

One-hundred-thirty-three consecutive FFPE tissue samples from 131 patients with suspected B-cell lymphoproliferative disorders (mostly skin biopsies) were received between 2000–2001 at Montefiore Medical Center, Department of Pathology, Bronx, NY. Three samples were excluded from this study: one had less than 2% B cells, and two skin biopsies inked with dibromohydroxymercu-

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ri fluorescein produced a highly fluorescent background. Therefore, we retrospectively analyzed 130 samples from 128 patients. We compared two different analytical methods for identifying monoclonal IgH gene rearrangement: an established, standard method using polyacrylamide gel electrophoresis (PAGE) *versus* our proposed new strategy using DNA melting curve analysis in the LightCycler System (Roche Molecular Biochemicals, Mannheim, Germany).

In addition, 23 fresh-frozen lymph nodes suspected of lymphoma were studied for IgH-R between 1995–1996 using Southern blot analysis, as previously described.¹² These archival DNA samples were also studied for IgH-R using melting curve analysis in the LightCycler System.

DNA Extraction

DNA was extracted from FFPE tissue samples according to standard methods. Briefly, twenty 5 μ m-thick sections were deparaffinized with xylene, washed twice with 100% ethanol, then vacuum-dried. DNA was reconstituted with PK buffer (100 mmol/L Tris-HCl, 4 mmol/L EDTA, pH 7.6) and digested with proteinase K at 56°C for 24 to 48 hours. Wizard PCR Preps DNA Purification System (Promega, Madison, WI) was used according to the manufacturer's recommendation and the concentration of DNA was determined with a DYNA-Quant minifluorometer (Hoefer, San Francisco, CA).

PCR Primers

The DNA from these samples was PCR-amplified using a commercial master mixture containing seven IgH variable gene framework region 3 (FR3) primers plus a joining region (JH) consensus primer (InVivoScribe Technologies, San Diego, CA). These primers have been designed to target conserved DNA sequences surrounding the unique hyper-variable antigen-binding complementarity determining region 3 (CDR3).¹³

Perkin-Elmer Thermalcycler PCR and PAGE Analysis

We used the IgH FR3 Master Mixture (InVivoScribe Technologies) to amplify the IgH variable region in a 9700 Perkin-Elmer thermalcycler, GeneAmp PCR System (Cetus, Norwalk, CT). Genomic DNA (500 ng) was added up to a final reaction volume of 50 μ l. After an initial "hot start" using TaqStart Antibody (Clontech Laboratories, Inc., Palo Alto, CA) at 95°C for 10 minutes, PCR cycle parameters were: denaturing at 94°C for 30 seconds, followed by annealing at 55°C for 30 seconds, and extending at 72°C for 1 minute. This program was repeated for 35 cycles. A final extension was performed at 72°C for 10 minutes. For PAGE analysis, 20 μ l of amplicon was electrophoresed on an 8% non-denaturing acrylamide gel (16 $\times$ 16 $\times$ 0.1 cm) using D Gene System (Bio-Rad Laboratories, Hercules, CA) at room temperature for 150 minutes at 200 volts. The gel was then stained with 0.5%

ethidium bromide and photographed under ultraviolet light. The expected size range of the amplicon varied from 69 to 129 bp. The quality of the DNA in each sample was confirmed by amplifying a 324-bp fragment of β -globin.¹⁴

LightCycler PCR and Melting Curve Analysis

The IgH variable region was amplified in the LightCycler System. The reaction mixture of each microcapillary contained 18 μ l of IgH FR3 Master Mixture, 0.4 μ l of TaqDNA polymerase (Promega), 0.2 μ l of 1:1000 dilution of SYBR Green I dye (Sigma) in TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 7.6), 0.1 μ l of bovine serum albumin (20 mg/ml; Sigma), and 0.8 μ l of 25 mmol/L MgCl₂ (Promega). Genomic DNA (100 ng) was added up to a final volume of 20.5 μ l. After initial "hot start" using TaqStart Antibody (Clontech Laboratories, Inc.) at 95°C for 10 minutes, PCR cycle parameters were: denaturing at 95°C for 0 second, annealing at 55°C for 10 seconds and extension at 72°C for 15 seconds. After 40 PCR amplification cycles, the LightCycler System DNA melting curve analysis was performed. In this program, PCR products were denatured at 95°C for 30 seconds, allowed to anneal at 55°C for 30 seconds and quickly raised to 70°C at a transition rate of 20°C/second; then the temperature was increased slowly from 70°C to 95°C at a transition rate of 0.05°C/second during continuous fluorescence monitoring at 521 nm.

Results

Optimization of DNA Melting Curve Analysis

We first determined the optimal number of PCR cycles needed to amplify IgH in the LightCycler System. Samples containing 100 ng of DNA from 100% Namalwa B-cell line, a mixture of 50% Namalwa and 50% tonsil, 100% tonsil, and a negative control containing all reagents without DNA were amplified for 20, 30, 40, 50, and 60 cycles (Figure 1). The ability to visualize the T_m inflection point of melted DNA was enhanced by plotting the negative first derivative ($-dF/dT$) *versus* T (F = fluorescence, T = temperature) as recommended by the manufacturer.

To select an optimal number of PCR cycles, we examined the $-dF/dT$ peak height ratio between Namalwa B-cell line and tonsil. The 100% Namalwa/100% tonsil $-dF/dT$ peak height ratio equaled 2.27 at 30 cycles and 3.88 at 40 cycles. Additional cycles did not significantly improve this ratio (Figure 1 and Table 1). Based on these observations, we set the criteria for diagnosing a monoclonal IgH-R DNA sample as having a $-dF/dT$ peak height at least twice as high, and less than one-half as wide as a polyclonal tonsil DNA sample when measured at the base of the curve after 40 PCR cycles.

Precision of LightCycler System Melting Curve Analysis

The precision of the method was tested by repeating the LightCycler PCR and DNA melting curve assay 10 sep-

Figure 1. Optimization of PCR cycles. DNA from 100% Namalwa B-cell line (N, **top curve**), a mixture of 50% Namalwa and 50% tonsil (0.5 N + 0.5 T, **middle curve**), and 100% tonsil (T, **bottom curve**) was amplified for 20 (**A**), 30 (**B**), 40 (**C**), 50 (**D**), and 60 (**E**) cycles. Namalwa started to produce a sharp $-dF/dT$ peak after 30 PCR cycles (**B**). The 100% Namalwa/100% tonsil $-dF/dT$ peak height ratio reached 3.88 at 40 cycles (**C** and Table 1). Additional cycles did not significantly improve this ratio. Therefore, we set the criteria for diagnosing a monoclonal B-cell DNA sample as having a $-dF/dT$ peak height at least twice as high, and less than one-half as wide, as a polyclonal tonsil DNA sample after 40 cycles. x axis = temperature ($^{\circ}\text{C}$); y axis = $-dF/dT$, where F = fluorescence and T = temperature.

arate times using 50% Namalwa B-cell line DNA diluted into tonsil DNA. The coefficient of variation (CV) of the assay was 0.47% at mean T_m equal to 84.98°C .

Reproducibility of Melting Curve

Repeated melting curve analyses of the same B-cell line and patient clonal DNA samples reproducibly yielded identical $-dF/dT$ versus T curves (low SD), indicating specific homoduplex formation. For example, triplicate repeat assays of two different monoclonal rearranged DNA samples yielded T_m values (mean $\pm$ SD) of 86.54 ± 0.06 and $85.68 \pm 0.05^{\circ}\text{C}$. On the other hand, repeated melting curve analysis of the same tonsil and patient polyclonal DNA samples produced different $-dF/dT$ versus T curves each time (high SD), indicating random formation of heteroduplexes. For example, triplicate repeat assays of two different patient polyclonal DNA samples yielded T_m values (mean $\pm$ SD) of 85.99 ± 0.79 and

$86.12 \pm 0.54^{\circ}\text{C}$. Thus, the SD of a polyclonal DNA sample is approximately tenfold greater than that of a monoclonal DNA sample.

Lower Limit for Detecting Monoclonal IgH Gene Rearrangement

To determine the minimum detectable percent clonal B-cell DNA with T_m analysis, we serially diluted Namalwa B-cell line (50%, 25%, 12.5%, 6.25%, 3.125%, and 1.56%) into tonsil. After performing PCR amplification for 40 cycles in the LightCycler, we could still detect a distinct peak by melting curve analysis with the expected T_m of the original B-cell clone at the 12.5% level (Figure 2).

Resolution of Melting Curve Analysis

To test whether melting curve analysis could discriminate the presence of two separate B-cell clones, equal

Table 1. Optimization of LightCycler PCR Cycle Number

Cycles	$-dF/dT$			$-dF/dT$ Ratio	
	Tonsil	50% Namalwa	100% Namalwa	50% Namalwa Tonsil	100% Namalwa Tonsil
20	0.06	0.09	0.11	1.51	1.83
30	0.33	0.52	0.75	1.58	2.27
40	0.32	0.92	1.24	2.88	3.88
50	0.38	1.08	1.55	2.84	4.08
60	0.35	1.29	1.64	3.68	4.68

$$-dF/dT \text{ ratio} = \frac{-dF/dT \text{ peak height of Namalwa B-cell line}}{-dF/dT \text{ peak height of tonsil}}$$

Figure 2. Minimal detection of B-cell clonality. Namalwa B-cell line DNA (50%, 25%, 12.5%, 6.25%, 3.13%, and 1.56%) were serially diluted into tonsil DNA. After 40 cycles of amplification in the LightCycler System, a distinct peak with the expected T_m of Namalwa was still detectable at the 12.5% level by DNA melting curve analysis. Axes definitions are the same as in Figure 1.

amounts of two different DNA samples were mixed together, PCR-amplified and analyzed using melting curve analysis in the LightCycler System (Table 2). We were able to resolve two peaks with a T_m difference equal to 1.78°C or greater. We could not resolve peaks with T_m difference of 0.49°C or 1.41°C. These data suggest that

two closely related biallelic populations with one or only a few base differences could not be resolved by the current assay. However, even unresolved mixed DNA samples (Table 2, experiments 1 and 2) yielded a single sharp peak with an intermediate T_m value that was clearly distinguishable from a broad polyclonal result.

Table 2. Melting Curve Analysis of Two Different DNA Samples

Experiment no.	Separate			After mixing*		
	T_m1	T_m2	ΔT_m	T_m1	T_m2	ΔT_m
1	86.16	86.65	0.49	86.51	—	—
2	85.36	86.77	1.41	86.31	—	—
3	86.68	88.46	1.78	86.39	88.31	1.92
4	86.11	88.49	2.39	85.78	88.25	2.47
5	84.94	87.57	2.63	84.71	87.32	2.61

*Equal amounts of two different DNA samples were mixed together, PCR-amplified and analyzed using melting curve analysis in the LightCycler. —, second peak not resolved.

Table 3. Summary of Cases Analyzed by PAGE and LightCycler

Patient	Age/sex	Tissues	Diagnosis	DNA sample	Tm
1	76/M	Skin	LPL	1*	82.21
1	76/M	Skin	LPL	2*	82.23
2	74/F	Skin	MZL	3	82.83
3	80/F	Skin	FL	4	83.26
4	67/F	Skin	FL	5	84.08
5	36/M	Skin	MZL	6	84.17
6	63/F	Skin	FL	7	84.37
7	79/F	Skin	MZL	8	84.41
8	81/F	Skin	MZL	9	84.48
9	82/F	Skin	LG	10	84.51
10	71/M	Parotid	MZL	11	84.66
11	87/F	Skin	DLBCL	12	84.69
12	85/M	Skin	FL	13	84.91
13	36/M	Skin	MZL	14	84.96
14	47/F	Tonsil	MZL	15	85.16
15	59/M	Lymph node	MZL	16	85.23
16	88/F	Skin	MZL	17	85.34
17	70/M	Skin	DLBCL	18	86.11
18	47/M	Lymph node	MCHL	19	86.18
19	71/M	Skin	FL	20	86.72
20	77/F	Skin	DLBCL	21	86.92
21	79/M	Skin	FL	22**	87.03
21	79/M	Skin	FL	23**	87.03
22	86/M	Skin	CLL	24	87.17

In two patients, two different biopsies were studied: samples 1 and 2 came from patient 1*; samples 22 and 23 came from patient 21**. In each, the DNA melting curves yielded essentially identical Tm values for the first and second biopsies, proving the persistence of the original B-cell clone.

CLL, B-cell chronic lymphocytic leukemia; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; LPL, lymphoplasmacytic lymphoma; LG, lymphomatoid granulomatosis; MCHL, mixed cellularity Hodgkin lymphoma; MZL, marginal zone B-cell lymphoma.

Comparison of PAGE and Melting Curve Analysis

We compared PAGE *versus* melting curve analysis for detecting monoclonal IgH-R in 130 DNA samples from 128 patients with suspected B-cell lymphoproliferative disorders. The identical, commercially available, IgH FR3 and JH primers were used to amplify the DNA for both analytic techniques (see Materials and Methods). In the gel electrophoresis method, a clonal IgH-R was defined as a sharp band on an ethidium bromide-stained acrylamide gel. In the melting curve analysis, we used SYBR Green I fluorescent dye, which intercalates into double-stranded DNA, for measuring a sharp decrease in fluorescence as the sample is heated through its Tm to form single-stranded DNA. A clonal IgH-R was defined as a narrow DNA melting curve (plotted as $-dF/dT$ *versus* T) that was greater than twice the peak height, but less than one-half the width, of a polyclonal B-cell tonsil sample (Figure 1).

Among 130 DNA samples tested, we detected monoclonal IgH-R in 24 samples from 22 patients (Table 3). These cases included 20 skin biopsies, 2 lymph nodes, 1 tonsil and 1 parotid gland. The morphological diagnoses were: extranodal marginal zone B-cell lymphoma (MZL) (N = 8); follicular lymphoma (N = 7); diffuse large B-cell lymphoma (N = 3); lymphoplasmacytic lymphoma (N =

2); nodal MZL (N = 1); lymphomatoid granulomatosis (N = 1); mixed cellularity Hodgkin lymphoma (MCHL) (N = 1); and B-cell chronic lymphocytic leukemia (N = 1). The single case of MCHL had numerous (greater than 5%) Reed-Sternberg cells. Melting curve analysis using the LightCycler System, compared to PAGE, revealed no false negative and no false positive results, yielding both sensitivity and specificity equal to 100%.

In two patients, DNA samples from two different biopsies yielded identical band patterns by PAGE and identical Tms by DNA melting curve analysis (Figure 3 and Table 3). We interpreted these results as reflecting persistence of the same B-cell clone. The Tms from 24 B-cell clonal clinical samples plus two B-cell lines (Namalwa and Ramos) ranged between 82°C to 88°C, with median Tm equal to 84°C (Table 3). Since the primer-dimer Tm was always less than 70°C, it did not interfere with determining monoclonal IgH-R in any case.

Comparison of Southern Blot Analysis and Melting Curve Analysis

We also compared Southern blot *versus* melting curve analysis for detecting IgH-R in 23 clinical DNA samples from fresh-frozen lymph nodes of 23 patients. We detected IgH-R by melting curve analysis in 7 DNA samples (Table 4). Melting curve analysis, compared to Southern blot analysis, revealed sensitivity equal to 58.3% (7 of 12) and specificity equal to 100% (11 of 11) (Table 5). The relatively low sensitivity is not surprising given the high proportion of follicular lymphomas in our samples, which are known to be hypermutated. However, our results closely match the sensitivity achieved by PCR in a recent multi-center study.¹⁵

Discussion

The IgH variable (V), diversity (D), and joining (J) gene segments recombine during early B-cell development: nucleotides may be randomly deleted or inserted in the V-D and D-J joining sites.¹⁶ Later, when the B cell enters the germinal center, the somatic mutation mechanism further enhances diversity.¹⁶ The resulting unique DNA sequence, CDR3, creates a B-cell signature that has been exploited to detect clonal IgH gene rearrangements.¹⁶⁻¹⁸ Current DNA-based PCR strategies for detecting monoclonal IgH gene rearrangement measure either the length of the DNA product, such as PAGE and capillary gel electrophoresis (CGE),^{4,5,15} or gel mobility variations arising from conformational changes, such as single-stranded conformation polymorphism analysis (SSCP) and denaturing gradient gel electrophoresis (DGGE).^{3,6,7}

We hypothesized that internal melting domains found in CDR3 could be used as a fingerprint for identifying B-cell clones. IgH FR3 and JH consensus primers yield PCR products with similar 5' and 3' ends but unique internal sequences, particularly across the V-D and D-J junctions and the D segment that define CDR3.^{17,18} Since

Figure 3. Detecting B-cell clonality in representative clinical DNA samples using melting curve analysis. **Sharp peaks** above **dashed line** ($-dF/dT = 0.35$) indicate clonally rearranged IgH: Namalwa B-cell line and four-patient DNA samples^{1,2,5,19} from three patients (see Table 2). DNA samples 1 and 2, obtained from a single individual, share the same T_m value indicating clonal identity. **Broad peaks** below **dashed line** ($-dF/dT = 0.35$) indicate polyclonal IgH rearrangements: tonsil and five unnumbered patient DNA samples. Axes definitions are the same as in Figure 1.

Table 4. Summary of Cases Analyzed by Southern Blot and LightCycler

Patient	Age/sex	Lymph node	Diagnosis	DNA sample	T_m
1	78/F	Cervical	FL	1	84.02
2	57/M	Axillary	FL	2	85.22
3	52/M	Supraclavicular	FL	3	85.31
4	46/M	Inguinal	FL	4	85.84
5	61/M	Axillary	FL	5	85.93
6	60/M	Axillary	MCL	6	86.61
7	55/F	Inguinal	FL	7	86.62
8	53/M	Supraclavicular	FL	8	—
9	56/M	Cervical	FL	9	—
10	65/F	Axillary	FL	10	—
11	78/F	Cervical	DLBCL	11	—
12	82/F	Supraclavicular	FL	12	—

DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; MCL, mantle cell lymphoma. —, Broad peak ($-dF/dT$ vs. T).

mismatches at the internal melting domains are more destabilizing than mismatches at the ends, one expects that heteroduplex amplicons to melt at a lower T_m than homoduplex amplicons.^{19–21} Although there has been a recent report of applying an analogous method for detecting clonal T-cell receptor γ gene rearrangement,²² to our knowledge, we are the first to experimentally demon-

Table 5. IgH Gene Rearrangement Test Results Obtained Using Southern Blot and LightCycler

		Southern blot		
		Not		Total
		Rearranged	rearranged	
LightCycler	Rearranged	7	0	7
	Not rearranged	5	11	16
Total		12	11	23

Sensitivity, 58.3%; specificity, 100%.

strate that PCR amplicons from monoclonal B cells have a higher and sharper $-dF/dT$ peak, reflecting homoduplex formation, compared with polyclonal B cells, which tend to have a much lower and broader $-dF/dT$ peak, reflecting heteroduplex formation (Figures 1 to 3).

The general method that we have described using consensus FR3 and JH primers is clearly not suitable for detecting minimal residual disease (MRD) since our sensitivity limit was only 12.5%. Further, small samples partially involved by monoclonal B-cell proliferations would also likely be missed. Other investigators have used either clone-specific primers or allele-specific oligonucleotide probes in a LightCycler format for quantifying MRD, with sensitivity yielding between 10^{-4} and 10^{-6} leukemic cells.^{23,24}

When we compared Southern blot analysis with the LightCycler method for IgH-R from lymph node DNA samples (mostly follicular lymphomas), we achieved sensitivity equal to 58.3%, which is a typical sensitivity for PCR-based assays.¹⁵ An additional set of FR primers would likely improve these results, as recently demonstrated using capillary electrophoresis.⁵

DNA melting curve analysis in the LightCycler System has several advantages for determining monoclonal IgH-R: monoclonal *versus* polyclonal B cells are distinguished based on a fundamental DNA characteristic;⁹ very fast temperature transition rates give rapid PCR results (approximately 40 minutes);²⁵ precise temperature control produces accurate and reproducible Tms;²⁵ combined PCR and DNA melting curve analysis in a closed system reduce cross-contamination risk; and 100% sensitivity and 100% specificity, compared to PAGE, in 130 clinical FFPE tissue samples (mostly skin biopsies) and 58.3% sensitivity and 100% specificity, compared to Southern blot, in 23 fresh-frozen lymph nodes (mostly follicular lymphomas) strongly suggest that DNA melting curve analysis in the LightCycler System has potential for evaluating monoclonal IgH-R in a clinical environment.

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