Rapid Detection of Clonal T-Cell Receptor- β Gene Rearrangements in T-Cell Lymphomas Using the LightCycler-Polymerase Chain Reaction with DNA Melting Curve Analysis

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Various molecular methods have been developed to diagnose clonal T-cell receptor (TCR) gene rearrangements in clinical samples. Most polymerase chain reaction strategies for detecting clonal TCR gene rearrangements rely on either gel or capillary electrophoresis. However, a cumbersome manual transfer step separates amplification from analysis. Recently, we developed a novel polymerase chain reaction assay using the LightCycler system to detect clonal immunoglobulin heavy chain gene rearrangement. In the current study, we extend this work to include the TCR. We report that clonal TCR- β (TCR- β) gene rearrangements can be detected in less than 1 hour after preparing the DNA by measuring DNA melting immediately after amplification in a single closed capillary tube. We retrospectively studied 52 fresh-frozen tissue samples from patients clinically suspected of T-cell malignancy. A clonal TCR- β gene rearrangement was detected in 14 samples by DNA melting curve analysis. When DNA melting was compared to the gold standard methods of Southern blot or denaturing gradient gel electrophoresis, it achieved a sensitivity equal to 71% and a specificity equal to 94%. We also compared melting curve analysis and polyacrylamide gel electrophoresis: melting curve analysis reached a sensitivity equal to 100% and a specificity equal to 97%. We conclude that DNA melting curve analysis in the LightCycler system has potential for clinical use as a new, ultra-fast method for the initial diagnosis of clonal TCR- β gene rearrangements. (*J Mol Diagn* 2005, 7:81–88)

Southern blot analysis remains the gold standard for detecting T-cell antigen receptor (TCR) gene rearrangements;¹ it usually targets the TCR- β gene because the rearrangements of TCR- γ are too limited to reliably dis-

tinguish monoclonal from polyclonal T-cell populations. But because the polymerase chain reaction (PCR) is rapid and technically robust, it has gradually replaced Southern blot analysis as the method of choice for detecting clonal TCR gene rearrangements.^{2–4}

The TCR- γ gene has become a favorite target for PCR-based T-cell clonality assays for two reasons: it is rearranged in most α/β and γ/δ T cells and its structure, containing 14 V segments and 5 J segments, is relatively simple. In contrast, the TCR- β locus is highly complex: it includes 64 to 67 V segments, 2 D segments, and 13 J segments.^{5–7} Thus, there have been few diagnostic PCR studies of TCR- β gene arrangements compared to TCR- γ because of the necessity for many primers. But now a set of rigorously tested multiplex primers for TCR- β are available.⁸

Traditional strategies for detecting TCR clonality using DNA-based PCR rely either on measuring the length of the amplicon^{9–14} or on detecting gel mobility variations owing to sequence-dependent conformational changes.^{15–20} Recently, we and others have shown that DNA melting curve analysis is a rapid and accurate method for detecting clonal immunoglobulin heavy chain (IGH) gene rearrangements.^{21–23} Melting curve analysis has also proven to be useful for studying clonal TCR- γ gene rearrangements.²⁴ Because TCR- β is structurally similar to IGH in terms of containing not only V and J but also D gene segments,²⁵ we hypothesized that DNA melting curve analysis could detect clonal TCR- β gene rearrangements as well. Therefore, we evaluated the diagnostic utility of combining TCR- β PCR and melting curve analysis in a panel of T-cell malignancies and reactive lymphoid tissues.

Materials and Methods

Patient Samples

We retrospectively analyzed both archival fresh-frozen and formalin-fixed, paraffin-embedded (FFPE) DNA samples that were obtained from patients clinically suspected

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of malignant lymphoma. The fresh-frozen DNA was mostly from lymph nodes and the FFPE DNA was mostly from skin biopsies that had been collected between 1996 to 2003 and stored at the Department of Pathology, Montefiore Medical Center, Bronx, NY. We assayed TCR- β gene rearrangements using the LightCycler (Roche Molecular Biochemicals, Mannheim, Germany) in 52 fresh-frozen DNA samples and in 42 FFPE DNA samples. Previously, the fresh-frozen DNA samples had been studied by only TCR- β Southern blot ($n = 33$), by only TCR- γ PCR-denaturing gradient gel electrophoresis (DGGE) ($n = 8$), or by both TCR- β Southern blot and TCR- γ PCR-DGGE ($n = 11$). In contrast, all 42 FFPE DNA samples had been studied by TCR- γ PCR-DGGE by the method of Greiner and colleagues,¹⁶ but none had been studied by TCR- β Southern blot. We compared two different strategies for diagnosing T-cell clonality: the established, gold standard, gel-based methods using either Southern blot or PCR-DGGE versus a new approach using DNA melting curve analysis in the LightCycler system.

DNA Extraction

For fresh-frozen tissue, DNA was extracted according to the manufacturer's instructions using the Puregene genomic DNA purification kit (Gentra System, Minneapolis, MN). For FFPE tissue, 20 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 ethylenediaminetetraacetic acid, pH 7.6) and digested with proteinase K at 56°C for 24 to 48 hours. The Wizard PCR Preps DNA purification system (Promega, Madison, WI) was used to purify the DNA, and the concentration of DNA was determined with a Dyna-Quant minifluorometer (Hoefer, San Francisco, CA).

PCR Primers

In the experiments using melting curve analysis in the LightCycler, the DNA was PCR-amplified using a commercial TCR- β gene clonality assay kit containing three master mixtures (BIOMED-2; InVivoScribe Technologies, Carlsbad, CA). These primers have been designed to target conserved V, D, and J DNA sequences that surround the hypervariable region (complementary determining region 3; CDR3): primer set A contains 23 V β , 6 J β 1, and 3 J β 2 primers; primer set B contains 23 V β and 4 J β 2 primers; primer set C contains 2 D β and 13 J β primers.⁸ For the experiments using DGGE, the TCR- γ V-J DNA sequences were PCR-amplified using published primers recognizing V γ 1 to V γ 8, V γ 9, V γ 10, V γ 11, and J γ 1/2, J γ P, J γ P1/P2.¹⁶

Southern Blot, Perkin-Elmer Thermalcycler PCR, and DGGE Analysis

Detection of TCR- β clonality using Southern blot analysis was performed as previously described using a radioac-

tively labeled J region probe.²⁶ Detection of TCR- γ using DGGE was based on the method described by Greiner and colleagues.¹⁶ Briefly, TCR- γ primers were used to amplify the TCR- γ variable region in a 9700 Perkin-Elmer thermalcycler, GeneAmp PCR system (Cetus, Norwalk, CT). The acrylamide gel gradient was 10 to 50% formamide. The quality of the DNA in each sample was confirmed by amplifying a 324-bp fragment of β -globin.²⁷

LightCycler PCR and DNA Melting Curve Analysis

The target regions of TCR- β were amplified in the LightCycler. The reaction mixture of each microcapillary contained 18 μ l of TCR- β Master Mixture (primer set A, B, or C; InVivoScribe Technologies), 0.4 μ l of TaqDNA polymerase (Promega, Madison, WI), 0.2 μ l of 1:400 dilution of SYBR Green I dye (Sigma, St. Louis, MO) in TE buffer (10 mmol/L Tris-HCl, 1 mmol/L ethylenediaminetetraacetic acid, 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 21.5 μ l. After initial hot start using TaqStart Antibody (Clontech Laboratories, Inc., Palo Alto, CA) at 95°C for 10 minutes, PCR cycle parameters were: denaturing at 95°C for 5 seconds, annealing at 60°C for 20 seconds for primer set A or at 65°C for 20 seconds for primer set C, and extension at 72°C for 30 seconds. We were unable to find suitable conditions for using primer set B. After 50 PCR amplification cycles, the LightCycler 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 65°C at a transition rate of 20°C per second; then the temperature was increased slowly from 65°C to 96°C at a transition rate of 0.05°C per second during continuous fluorescence monitoring at 521 nm. DNA melting curve analysis was repeated several times after PCR amplification.

LightCycler PCR and Polyacrylamide Gel Electrophoresis (PAGE)

After recording the melting curve, 10 μ l of LightCycler PCR reaction product using primer set C was electrophoresed on a 6% 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 V. The gel was then stained with 0.5% ethidium bromide and photographed under ultraviolet light. The expected size ranges of the amplicons were either 170 to 210 or 285 to 325 bp.

Results

Optimization of DNA Melting Curve Analysis

We determined the optimal number of PCR cycles needed to amplify TCR- β in the LightCycler system. Samples containing 100 ng of DNA from 100% Jurkat T-cell

Figure 1. Optimization of PCR cycles. DNA samples from 100% Jurkat T-cell line (J, **top curve**), a mixture of 50% Jurkat and 50% tonsil (0.5 J + 0.5 T, **middle curve**), 100% tonsil (T, **bottom curve**), and a negative control including all reagents without DNA (H₂O) were amplified for 20 (A), 30 (B), 40 (C), 50 (D), and 60 (E) cycles. **B:** Jurkat started to produce a sharp $-dF/dT$ peak after 30 PCR cycles. **D:** The $-dF/dT$ peak height ratio of 100% Jurkat/100% tonsil reached 4.1 at 50 cycles (Table 1). Additional cycles did not significantly improve this ratio. Therefore, the diagnosis of a clonal T-cell gene rearrangement sample was based on DNA melting after 50 PCR cycles. x axis = temperature, $^{\circ}\text{C}$; y axis = $-dF/dT$, where F = fluorescence and T = temperature.

line, a mixture of 50% Jurkat with 50% tonsil, 100% tonsil, and a negative control including 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. The $-dF/dT$ peak heights were measured, and the peak height ratios for 100% and for 50% Jurkat in reference to tonsil were compared (Table 1). We chose the maximal peak height ratio of Jurkat:tonsil at 50 PCR cycles, because a greater number of cycles did not improve the separation of monoclonal and polyclonal T-cell samples.

Precision of LightCycler System Melting Curve Analysis

The precision of the method was tested by repeating the LightCycler PCR and DNA melting curve assay 5 to 10 separate times using DNA extracted from Jurkat and CEM T-cell lines. The coefficient of variation of the assay is summarized in Table 2. The polyclonal tonsil background had essentially no influence on the T_m values of the clonally rearranged Jurkat T-cell line DNA when amplified with either primer set A or C (compare lines 1 and 2 in Table 2).

Table 1. Optimization of LightCycler PCR Cycle Number

Cycles	$-dF/dT$			$-dF/dT$ Ratio	
	Tonsil	50% Jurkat	100% Jurkat	50% Jurkat	100% Jurkat
				Tonsil	Tonsil
20	0.03	0.04	0.04	1.33	1.33
30	0.13	0.31	0.36	2.38	2.77
40	0.12	0.27	0.40	2.25	3.33
50	0.10	0.38	0.41	3.80	4.10
60	0.08	0.22	0.41	2.75	5.13

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

Table 2. Coefficient of Variation (CV) of LightCycler System Melting Curve Analysis

Cell lines	Primer set A			Primer set C		
	<i>n</i>	T _m , mean	CV	<i>n</i>	T _m , mean	CV
50% Jurkat	7	86.99	0.45	7	90.18	0.26
100% Jurkat	10	87.25	0.44	5	90.11	0.27
100% CEM	7	91.96	0.32	7	92.14	0.14

T_m = DNA melting temperature, °C. CV (%) = (SD/mean) × 100.

Analytic Sensitivity for Detecting Monoclonal TCR-β Gene Rearrangement

The analytic sensitivity was defined as the minimum percentage of clonal T cells that could be detected. We serially diluted DNA from Jurkat T-cell line (50%, 25%, 12.5%, 6.25%, 3.125%, and 1.56%) into DNA from tonsil and performed PCR amplification for 50 cycles in the LightCycler. We could still detect a distinct peak by DNA melting curve analysis with the expected T_m of the original T-cell clone at 12.5% using primer set A and at 6.25% using primer set C (Figure 2).

Resolution of Melting Curve Analysis

To test whether melting curve analysis could discriminate the presence of two separate T-cell clones, equal amounts of two different clonal DNA samples were mixed together, PCR-amplified with primer set A, and melting curve analysis was applied in the LightCycler System (Table 3). We could resolve two peaks with a T_m difference equal to 1.6°C or greater, but not with a T_m difference of 0.85°C. These data suggest that closely related

biclonal TCR-β gene rearrangements with 1 or only a few bp differences would not be resolved by this assay. However, even unresolved mixtures (Table 3, experiment 1) yielded a single sharp peak that was clearly distinguishable from a broad polyclonal result. We did not study the resolving power of this assay using primer set C because there was a very narrow T_m range of only 0.82°C (Table 4).

Sensitivity and Specificity of Melting Curve Analysis

We compared DNA melting curve analysis versus Southern blot or PCR-DGGE. The LightCycler TCR-β gene rearrangement assays were performed on 52 DNA samples extracted from fresh-frozen tissues and on 42 DNA samples extracted from FFPE tissues. In addition, we compared LightCycler PCR-PAGE versus DNA melting curve analysis. In the PAGE method, a clonal TCR-β gene rearrangement was defined as a sharp band on an ethidium bromide-stained acrylamide gel. In the DNA 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 T_m to form single-stranded DNA. In the LightCycler system, a clonal TCR-β gene rearrangement was defined as a narrow DNA melting curve (plotted as -dF/dT versus T) that was greater than one and one-half times the peak height of a polyclonal tonsil or a ΔT_m (sample T_m - tonsil T_m) equal to or greater than 1.5°C.

We detected a clonal TCR-β gene rearrangement in 14 of 52 DNA samples from fresh-frozen tissues using DNA melting curve analysis (primer sets A and C, Table 4). These cases included eight lymph nodes, one bone marrow, one spleen, one cerebrospinal fluid, one peripheral blood, one para-spinal mass, and one cervical mass. The diagnoses were: peripheral T-cell lymphoma (*n* = 6), T-cell anaplastic large cell lymphoma (*n* = 4), precursor T-lymphoblastic lymphoma (*n* = 1), Sézary syndrome (*n* = 1), composite T-cell and B-cell lymphoma (*n* = 1), and classic Hodgkin's lymphoma (*n* = 1). The remaining 38 cases were negative for TCR-β gene rearrangement by DNA melting curve analysis. These included reactive lymphoid hyperplasia (*n* = 12), B-cell lymphoma (*n* = 12), Hodgkin's lymphoma (*n* = 5), natural killer cell lymphoma (*n* = 3), panniculitis (*n* = 1), and T-cell lymphoma (*n* = 5). Among these 38 cases, there are 4 false-negative cases compared to TCR-β Southern blot and 3 false-negative cases compared to TCR-γ PCR-DGGE.

Figure 2. Minimal detection of percent clonal T cell. Jurkat T-cell line DNA (100%, 50%, 25%, 12.5%, 6.25%, and 3.125%) was serially diluted into tonsil DNA. After 50 cycles of amplification in the LightCycler system, we could still detect a distinct peak with the expected T_m of Jurkat by DNA melting curve analysis at the 12.5% level with primer set A (A) and at 6.25% level with primer set C (B).

Table 3. Melting Curve Analysis of Two Different DNA Samples

Experiment no.	Separate			After mixing*		
	Tm1	Tm2	ΔT_m	Tm1	Tm2	ΔT_m
1	87.72	86.87	0.85	86.52	—	—
2	88.47	86.87	1.60	88.52	86.44	2.08
3	92.44 [†]	87.68 [‡]	4.76	92.23	87.03	5.20

* Equal amounts of two different clonal DNA samples were mixed together, PCR-amplified with primer set A and analyzed using melting curve analysis in the LightCycler.

[†] CEM T-cell line.

[‡] Jurkat T-cell line.

—, Second peak not resolved.

Figure 3 illustrates examples of clonal TCR- β gene rearrangements that were detected by DNA melting curve analysis using primer sets A and C. The T_m of the clonal T-cell sample was always higher than the T_m of the tonsil in the same run. Because the T_m s of the primer-dimers were less than 82°C, they did not interfere with analyzing the DNA melting curves of the samples.

Because this was a retrospective study in which the Southern blot and DGGE analyses were performed months or years before the LightCycler analysis, we chose a limited number of newly amplified TCR- β PCR products to directly compare the sensitivity of PAGE versus DNA melting analysis on the identical PCR product. When the same LightCycler PCR product generated by primer set C was analyzed by both DNA melting curve and by PAGE, we obtained a sensitivity equal to 82% and a specificity equal to 97% (Table 5). However, because non-T-cell malignancies contributed two false-positive PAGE results, the actual sensitivity of DNA melting curve analysis versus PAGE was 100%. The two cases that were identified as false-positive results were negative for TCR- β gene rearrangements by Southern blot: the pathological diagnoses were reactive lymphoid hyperplasia (lymph node) and lymphomatoid granulomatosis (lung).

DNA melting curve analysis, using both primer sets A and C, compared favorably to Southern blot and/or PCR-

DGGE: sensitivity = 71%; specificity = 94% (Table 6A). LightCycler DNA melting yielded similar sensitivities and specificities when compared to Southern blot only (Table 6B) and to PCR-DGGE only (Table 6C). There were five T-cell malignancies in a subset of 11 cases that had been analyzed by Southern blot, PCR-DGGE, and DNA melting: clonal TCR gene rearrangements were detected in four, three, and three cases each (Table 6D). DNA melting curve analysis detected a clonal TCR- β gene rearrangement in one case of T-cell anaplastic large cell lymphoma that, because of a paucity of lymphoma cells, appeared to have a germline pattern by Southern blot.

We also compared PCR-DGGE for TCR- γ versus DNA melting curve analysis for TCR- β gene rearrangements among 42 clinical DNA samples extracted from FFPE tissues. Using primer set A, 5 of 42 samples (12%) failed to amplify in the LightCycler. Using primer set C, we did not succeed in detecting any clonal TCR- β gene rearrangements by DNA melting curve analysis in these samples. PCR-DGGE detected a clonal TCR- γ gene rearrangement in 29 of 37 cases. In contrast, DNA melting curve analysis detected a clonal TCR- β gene rearrangement in only 11 of these 29 cases (38%). Thus, FFPE tissue does not appear suitable for the LightCycler TCR- β gene rearrangement assay.

Table 4. Summary of Cases Analyzed by Southern Blot, DGGE, and LightCycler DNA Melting

Patient	Age/ sex	Tissues	Diagnosis	LightCycler DNA melting				Southern blot TCR- β	PCR- DGGE TCR- γ
				Primer set A		Primer set C			
				TCR- β	Tm	TCR- β	Tm		
1	58M	CSF	PTL	+	85.09	—			+
2	56F	PB	SS	+	85.46	—			+
3	50M	LN	T-MLLB	+	86.31	+	89.45	+	
4	49F	LN	ALCL	+	86.67	+	90.02	—	—
5	49M	LN	PTL	+	87.99	—		+	
6	84M	BM	Comp B+T	+	88.04	+	89.49		+
7	52M	PSM	ALCL	+	89.28	—		+	
8	37M	LN	ALCL	—		+	90.05	+	
9	77M	LN	PTL	—		+	89.50	+	
10	53F	LN	PTL	—		+	89.62	+	
11	69F	Spleen	PTL	—		+	89.83	+	+
12	75F	LN	ALCL	—		+	90.22		+
13	34M	LN	PTL	—		+	90.27		+
14	44F	LN	HL	—		+	90.15	—	

CSF, cerebrospinal fluid; PB, peripheral blood; LN, lymph node; BM, bone marrow; PSM, para-spinal mass; T-MLLB, precursor T-lymphoblastic lymphoma; SS, Sézary syndrome; PTL, peripheral T-cell lymphoma; ALCL, T-anaplastic large cell lymphoma; Comp T + B, composite T-cell and B-cell lymphoma; HL, Hodgkin lymphoma; PCR-DGGE, analysis of TCR- γ gene rearrangement by conventional polymerase chain reaction and denaturing gradient gel electrophoresis; +, clonal TCR gene rearrangement; —, nonclonal TCR gene rearrangement.

Figure 3. Detecting T-cell clonality in representative clinical DNA samples by melting curve analysis using primer set A (**A**) and primer set C (**B**). Sharp peaks above the **dashed line** ($-dF/dT = 0.85$ for **A** and 0.28 for **B**) indicate clonally rearranged TCR- β : Jurkat T-cell line (J), CEM T-cell line (C), and three patient DNA samples (**A** and Table 4) and four patient DNA samples (**B** and Table 4). Broad peaks under the **dashed line** indicate polyclonal TCR- β gene rearrangements in tonsil and two representative unnumbered patient DNA samples. Axes definitions are the same as in Figure 1.

Discussion

In the current report, we use LightCycler-PCR and DNA melting curve analysis to experimentally demonstrate that clonal TCR- β gene rearrangements in T-cell lymphomas produce homoduplex PCR amplicons with a sharp $-dF/dT$ peak. In contrast, nonclonal TCR- β gene rearrangements produce heteroduplexes in tonsil, B-cell lymphomas, and reactive lymphoid hyperplasia. To our knowledge, only one other LightCycler study has used DNA melting to analyze TCR gene rearrangements: clonal TCR- γ gene rearrangements were reported in a series of cutaneous T-cell lymphomas; the sensitivity equaled 59% by melting curve analysis and 72% by PAGE.²⁴

The sensitivity of a TCR- β gene rearrangement PCR assay is dependent on both the primers and the analytic system. For example, PCR-PAGE assays yield a sensitivity of 44 to 76%.^{28–30} On the other hand, two-step PCR

assays in combination with direct sequencing or seminested PCR in combination with GeneScan yield an enhanced sensitivity of 98 to 100%.^{2,19} However, all of these traditional methods require a cumbersome manual transfer step in between amplification and analysis.

The distribution of gene segments in either TCR- β or TCR- γ genes are highly variable. For the TCR- β gene, no single common sequence is sufficient to identify and amplify all of the possible rearrangements that occur in T-cell lymphomas.^{3,19,30} Therefore, multiple primer sets are needed. The same is true for the detection of TCR- γ gene rearrangements.³¹ Recently, standardized multiplex PCR primers have been tested in 32 diagnostic PCR laboratories in Europe (BIOMED-2 Concerted Action).⁸ In the TCR- β PCR portion of this study, clonal TCR- β gene rearrangements were detected by heteroduplex analysis in 86% of cases and by GeneScan analysis in 79% of cases of 29 Southern blot defined cases. A similar study in fresh-frozen tissues, using BIOMED-2 multiplex PCR primers, detected clonal TCR- β gene rearrangements using heteroduplex and GeneScan analyses in 76% and 66% of cutaneous T-cell lymphomas, which was comparable to Southern blot analysis (68%).³² In the current report, we have used the BIOMED-2 TCR- β primers for PCR amplification, but DNA melting for analysis. Overall, we achieved a sensitivity of 71%, which is equivalent to the other methodologies.

Although we could routinely amplify TCR- β using primer sets A and C, we were not successful using primer set B. Both primer sets A and B cover the identical 23 V β gene segments. The major difference between primer sets A and B is in the coverage of the J region. Primer set A contains six J β 1 primers and three J β 2 primers: J β 1.1 to 1.6, 2.2, 2.6, and 2.7. On the other hand, primer set B contains four J β 2 primers and no J β 1 primers: J β 2.1, 2.3, 2.4, and 2.5. Primer set C contains two D β primers and no V β primers: D β 1 and D β 2. In addition, primer set C contains all 13 J β indicated above.⁸ Therefore, the inability to amplify TCR- β gene rearrangements using primer set B appears to be because of the J β 2.1, 2.3, 2.4, and 2.5 DNA sequences. Thus, we will underestimate TCR- β gene rearrangements that use these J β 2 sequences.

Real-time PCR using allele-specific oligonucleotides in the LightCycler can be used to quantify minimal residual disease in acute lymphoblastic leukemia at the 10^{-4} and 10^{-6} level of leukemia cells.^{33,34} Clearly, the TCR- β LightCycler-PCR method described here is not suitable for detecting minimal residual disease because the lower limits varied between 12.5% and 6.25% of clonal DNA. Nevertheless, the detection limit for TCR- β is of the same order of magnitude as other melting curve assays for IGH (12.5%)²¹ and for TCR- γ (10%).²⁴

Unfortunately, we could not detect TCR- β gene rearrangements in FFPE tissues using LightCycler and DNA melting curve analysis. In our previous study of IGH, DNA melting curve analysis did successfully detect gene rearrangements from FFPE tissues, but the size of those PCR amplicons was much smaller, ranging from 69 to 129 bp.²¹ In contrast, the BIOMED-2 TCR- β primer sets generate larger PCR products. Primer set A: 240 to 285 bp; primer set B: 240 to 285 bp; primer set C: 170 to 210

Table 5. TCR- β Gene Rearrangement Test Results Obtained Using PAGE and DNA Melting with Primer Set C

	PAGE		
	Rearranged	Not rearranged	Total
DNA Melting			
Rearranged	9	1	10
Not rearranged	2	38	40
Total	11	39	50

The same PCR products were analyzed by both PAGE and DNA melting curve analysis: sensitivity, 82%; specificity, 97%.

Table 6. TCR Gene Rearrangement Test Results: LightCycler DNA Melting versus Southern Blot and/or PCR-DGGE

LightCycler		Rearranged	Not rearranged	Total	Sensitivity	Specificity
A: Southern blot and/or PCR-DGGE*						
Primer set A	Rearranged	6	1	7	35%	97%
	Not rearranged	11	34	45		
	Total	17	35	52		
Primer set C	Rearranged	8	2	10	47%	94%
	Not rearranged	9	33	42		
	Total	17	35	52		
Primer sets A and C	Rearranged	12	2	14	71%	94%
	Not rearranged	5	33	38		
	Total	17	35	52		
B: Southern blot						
Primer sets A and C	Rearranged	7	2	9	64%	94%
	Not rearranged	4	31	35		
	Total	11	33	44		
C: PCR-DGGE						
Primer sets A and C	Rearranged	6	1	7	67%	90%
	Not rearranged	3	9	12		
	Total	9	10	19		
D:	PCR-DGGE TCR- γ	Southern blot TCR- β		LC DNA melting TCR- β		
Rearranged	4	3		3		
Not rearranged	7	8		8		
Total	11	11		11		

* Southern blot only, $n = 33$; PCR-DGGE only, $n = 8$; both Southern blot and PCR-DGGE, $n = 11$. TCR- β analyzed by LightCycler DNA melting and Southern blot; TCR- γ analyzed by PCR-DGGE. A: LightCycler DNA melting versus Southern blot and/or PCR-DGGE. B: LightCycler DNA melting versus Southern blot. C: LightCycler DNA melting versus PCR-DGGE. D: Comparison of 11 cases that were all analyzed by PCR-DGGE, Southern blot, and LightCycler DNA melting.

and 285 to 325 bp. Initially, we considered that the most likely reason why TCR- β clonality could not be detected in the LightCycler from FFPE tissues was because of the larger PCR products of TCR- β compared to IGH.^{21,27} However, after successfully reamplifying a 324-bp fragment of β -globin using conventional PCR soon after the LightCycler analysis, we conclude that DNA integrity by itself is not a sufficient explanation. We can only speculate that whereas the FFPE DNA could be amplified by conventional PCR, it may be suboptimal for producing relatively large amplicons under the LightCycler conditions. Also, DNA extracted from FFPE tissue tends to be severely fragmented and might contain PCR inhibitors that do not affect conventional PCR but could be detrimental in the LightCycler.³⁵

We believe that DNA melting curve analysis for the detection of clonal TCR- β gene rearrangements has several unique advantages: monoclonal versus polyclonal T cells are distinguished based on fundamental DNA characteristic such as length, sequence, G:C content, and Watson-Crick base pairing;^{36,37} very fast temperature transition rates give rapid results (less than 1 hour after DNA preparation); precise temperature control produces accurate and reproducible Tms; and combined PCR and DNA melting curve analysis in a closed system reduce cross-contamination risk. Using the same PCR products, melting curve analysis versus PAGE revealed sensitivity equal to 100% and specificity equal to 97%. Further, melting curve analysis compared to gold standard methods of Southern blot and DGGE revealed sensitivity equal to 71% and specificity equal to 94%. These results are

within the typical range of most other methods for detecting PCR products.^{8,29,32} Because the sensitivity of detecting a T-cell clone diluted in tonsil DNA is between 6.25% and 12.5%, DNA melting curve analysis is clearly not suitable for assaying minimal residual disease in T-cell lymphomas. Nevertheless, we believe that this LightCycler DNA melting assay could play a role in rapidly evaluating clonal TCR- β gene rearrangements in initial fresh or frozen tissue samples.

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