Technical Advance

Simultaneous Evaluation of T- and B-Cell Clonality, t(11;14) and t(14;18), in a Single Reaction by a Four-Color Multiplex Polymerase Chain Reaction Assay and Automated High-Resolution Fragment Analysis

A Method for the Rapid Molecular Diagnosis of Lymphoproliferative Disorders Applicable to Fresh Frozen and Formalin-Fixed, Paraffin-Embedded Tissues, Blood, and Bone Marrow Aspirates

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Current polymerase chain reaction (PCR) methods for the molecular diagnosis of B- and T-cell lymphomas by determination of clonality of immunoglobulin heavy chain (IgH) and T-cell receptor- γ rearrangements and by detection of the chromosomal translocations t(14;18) and t(11;14), require several laborious and costly PCR assays for each of these diagnostic tests. We have developed a multiplex PCR assay for the simultaneous determination of B- and T-cell clonality and the detection of the chromosomal translocations t(14;18) and t(11;14) in a single reaction, using four-color fluorescence and automated high-resolution fragment analysis. The 26 primers combined in the multiplex PCR correspond to the sequences of >90% of the 69 variables and 6 join IgH genes and 100% of the T-cell receptor- γ variables and join genes that could participate in the respective rearrangements. In addition, they detect the major and the minor breakpoint regions of the t(14;18) and the major breakpoint region of the t(11;14), and amplify the β -globin gene as an internal control. The specificity of the multiplex PCR was confirmed by analysis of 39 T-cell lymphomas and 58 B-cell lymphomas, including 11 mantle cell lymphomas bearing the t(11;14) and 25 follicular lymphomas

bearing the t(14;18), with known rearrangements and/or translocations. Fifteen samples of reactive lymphadenitis remained negative. (*Am J Pathol* 2001, 159:2031–2043)

Methods for the molecular diagnosis of B- and T-cell non-Hodgkin lymphomas are based on determination of clonality of the respective antigen receptors and detection of specific chromosomal translocations.¹ Antigen receptors are assembled in the course of lymphocyte development with one of numerous variable gene (V) segments fused to one of the join gene (J) segments and the intervening DNA being spliced out. In some receptors, eg, the immunoglobulin heavy chain (IgH) receptor, an additional diversity (D) gene segment is fused between the V and J segments. During V(D)J assembly, nucleotides are randomly inserted between the gene segments. Rearranged antigen receptors therefore have individual V-(D)-J junctions that differ in V, (D), and J usage, in the breakpoints of their V and J segments, and in the intervening DNA sequences (the so-called N-sequences). Analysis of antigen receptors by polymerase chain reaction (PCR) techniques allows distinguishing between clonal populations of lymphoid cells, which is highly indicative for malignancy, and expansion of poly-

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clonal lymphocytes because of reactive processes. In the majority of normal and neoplastic B cells, the IgH locus on chromosome 14 (14q32.33) is rearranged. Similarly, the T-cell receptor- γ (TCR- γ) locus on chromosome 7 (7p15-p14) is rearranged in the majority of T cells. Specific chromosomal translocations are associated with specific disorders. The most common translocations in B-non-Hodgkin lymphoma are t(11;14) (q13;q32), associated with mantle cell lymphoma, and t(14;18) (q32;q21), specifically found in follicle center cell lymphoma and in a part of the diffuse large-cell lymphomas.

PCR methods for the determination of clonality by analysis of IgH and TCR- γ rearrangements are widely used. Conventional methods require several PCR assays for the analysis of the different IgH V framework regions and for the analysis of the different TCR- γ V and J genes. Similarly, singleplex PCR assays for the detection of the t(11;14) and the t(14;18) are performed, some requiring additional laborious procedures, eg, hybridization with specific probes. More recently, multiplex PCR assays for the detection of TCR- γ rearrangements^{2,3} have been reported. Some groups reported the use of high-resolution fragment analysis (HRFA), which greatly improves separation and visualization of PCR fragments, for the analysis of PCR-based diagnosis of lymphoproliferative disorders.⁴⁻¹¹ Consensus primers and one fluorescent dye were used to detect TCR- γ ^{9,10} or IgH rearrangements.⁷ Multiplex PCR for the detection of TCR- γ rearrangements^{5,6} and IgH rearrangements⁵ by separate PCR assays and using one fluorescent dye have been reported. A method for the detection of TCR- γ and IgH rearrangements and the t(14;18) by four-color fluorescence and HRFA was reported.⁸ The method required five different PCR assays, the resulting fragments could then be combined for HRFA.

We have developed a four-color multiplex PCR assay for the simultaneous determination of B- and T-cell clonality, the detection of the most frequent and diagnostically important chromosomal translocations t(14;18) and t(11;14), and the amplification of a control gene in a single reaction. Analysis is performed using automated HRFA and GeneScan analysis. The details of these diagnostic tests are explained below.

IgH Rearrangement

The IgH locus harbors 123 genes for the variable IgH region (V), 79 of them being pseudogenes.¹² Forty-two of the 44 functional V genes and 27 of the V pseudogenes have recombination signal sequences that maintain the first three nucleotides of the heptamer (CAC) and the fifth and sixth position of the nonamer (AA/GA), critical for efficient recombination, as well as the 23-bp spacer sequence. We have designed PCR primers for the framework 1 region (FR1) of the V genes, which detect 60 of the 69 V genes capable of efficient recombination. From the remaining nine V genes three are detected by the primers designed for the framework region 3 (FR3). The FR3 primers detect the majority of the V genes also detected by the FR1 primers. In addition, the melting temperature

of the FR3 primers allows hybridization to FR3 regions that bear point mutations with respect to the FR3 consensus sequences. Together, the FR1 and FR3 primers detect >90% of the V genes that could participate in an IgH rearrangement. Six functional and three pseudo join (J) genes are present on the IgH locus. The sequences of the J gene primers confirm to those of the six functional join genes, as the pseudogenes lack the requirements for efficient rearrangement.

t(11;14)

In the t(11;14) (q13;q32), the bcl-1 (cyclin D1) gene on chromosome 11 is juxtaposed to the IgH join genes on chromosome 14. The influence of the IgH join gene enhancers leads to overexpression of the cyclin D1 mRNA and protein. Deregulated cyclin D1 expression contributes to tumorigenesis as cyclin D1 promotes cell-cycle progression in G₁. The t(11;14) is highly specific for mantle-cell lymphomas. Fifty-five to 60% of the mantle-cell lymphomas bear a t(11;14) and in 80% of these, the breakpoints are localized in the major translocation cluster (MTC).¹³ PCR analysis with primers for the MTC detect the t(11;14) in 30 to 50% of the mantle-cell lymphomas.¹³⁻¹⁹ Reports on frequencies of breakpoints in the minor breakpoint region (detected by the probe p94) are controversial. According to most of the published studies, these translocations seem to be very rare.¹⁹⁻²¹ We designed primers for the MTC region that cover all published breakpoints in the MTC.

t(14;18)

The t(14;18) (q32;q21) fuses the bcl-2 gene on chromosome 18 to the IgH join genes on chromosome 14. This results in overexpression of the anti-apoptosis protein bcl-2. Cytogenetic studies showed the presence of a t(14;18) in ~70% of follicular lymphomas and in ~30% of diffuse large-cell lymphomas.²² Approximately two thirds of the breakpoints on chromosome 18 are clustered in an area called the major breakpoint region (Mbr), another 5% are found in the minor cluster region (mcr). The remaining breakpoints are spread in a larger region on chromosome 18 and are therefore not amenable to PCR analysis. Reported frequencies of t(14;18) detected in follicular lymphomas by molecular methods widely vary. Detection rates of PCR-based assays between 40 and 50% seem realistic.²²⁻²⁵ The primers used in our multiplex PCR detect t(14;18) with breakpoints in both the Mbr and the mcr regions.

T-Cell Receptor- γ

The TCR- γ locus consists of 14 variable (V) genes, 8 of them being pseudogenes, most of which could nevertheless be rearranged, and 5 join (J) genes. The primers designed for the multiplex PCR amplify all existing V and J genes, except the V1S1P gene, whose recombination signal sequences with a spacer sequence of only 11 bp will not participate in a rearrangement. T cells frequently

have rearranged TCR- γ loci on both alleles. This is in contrast to IgH rearrangements in B cells, in which allelic exclusion does not allow rearrangement of the IgH locus on the second allele.

The limited diversity of the TCR- γ VJ combinations and the presence of relatively short N regions results in a small size range of the PCR fragments derived thereof. This may cause difficulties to discriminate clonal gene rearrangements from the polyclonal background caused by the considerable amount of nonneoplastic T cells frequently present in clinical samples of T-cell lymphomas. A number of methods have been suggested that improve the separation and visualization of the PCR fragments, eg, fragment analysis by denaturing gradient gel electrophoresis,^{26–29} single-strand conformation polymorphism analysis,^{30–32} temperature gradient gel electrophoresis,³³ and automated HRFA.^{4,6,34} The excellent resolution by HRFA will reveal clonal rearrangements in most cases. To provide security in cases with high polyclonal background, we have developed an additional four-color multiplex PCR assay for the verification of clonal TCR- γ rearrangements. This PCR assay further separates fragments derived from TCR- γ rearrangements by different colors according to the subtype-specific V gene present, and allows verification of clonal bands according to subtype-specific differences in the lengths of the fragments obtained in the first multiplex PCR assay and those obtained in the TCR- γ -specific PCR assay.

The 26 primers present in the multiplex PCR are labeled with three different fluorophores (5-FAM, 6-JOE, and NED) and the PCR fragments derived from the four different diagnostic tests are within defined size ranges. Excellent resolution is provided by automated HRFA performed by capillary electrophoresis and laser-induced fluorescence detection on an ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA), an automated instrument system capable of determining size and quantity of DNA fragments. Electrophoresis is performed in small, polymer-filled capillary tubes instead of flat gels. The dye-labeled PCR fragments electrophorese through the polymer and separate according to size. The fluorescent dyes are excited by a laser and emit light at a specific wavelength for each dye. The light is collected and separated by a spectrograph and collected onto a charge-coupled device camera. The collected data are automatically analyzed using the GeneScan Analysis software, which sizes the DNA fragments based on a size standard labeled with a forth fluorophor (ROX). PCR fragments differing in length by 1 bp can be distinguished. Results can be displayed as electropherograms, with are shown in separate panels for each dye, and as tabular data. The DNA fragments are quantified according to the intensity of the signal, which corresponds to the peak area.

A schematic representation of the fluorescent dye labels and the size ranges specific for the PCR fragments derived from each of the diagnostic tests covered by the multiplex PCR is shown in Figure 1. In Figure 1, panel B (blue), the fragments labeled with 6-FAM are shown. Size ranges are as follows: fragments of IgH rearrangements

Figure 1. Schematic representation of the fluorescent dye labels and the size ranges specific for the PCR fragments derived from each of the diagnostic tests. **Panel B:** (blue, label 6-FAM); IgH rearrangement fragments VFR3-J appear in a size range between 80 and 140 bp. Fragments of TCR- γ rearrangements appear in a size range between 180 and 280 bp. IgH rearrangement fragments V FR1-J are present in a size range between 300 and 400 bp. **Panel G:** (green, label 6-JOE); t(11;14) Mbr fragments have sizes between 160 and 300 bp. The control PCR fragment of the β -globin gene has a length of 352 bp. **Panel Y:** (yellow, label NED); the size of t(14;18) Mbr and mcr fragments is usually between 150 and 300 bp and may, in a rare case, be up to 500 bp. **Panel R:** (red, label ROX); molecular weight marker. Fragment sizes are indicated below.

amplified with primers for the V framework region 3 (FR3) are present in a size range between 70 and 140 bp. Fragments of TCR- γ rearrangements appear in a size range between 180 and 280 bp. Fragments of IgH rearrangements containing the V framework region 1 (FR1) are present in a size range between 300 and 400 bp. In Figure 1, panel G (green), the fragments labeled with 6-JOE are as follows: fragments derived from a t(11;14) MTC have sizes between 160 and 300 bp. The length of the fragment of the control PCR (β -globin) is 352 bp. Figure 1, panel Y (yellow), shows fragments labeled with NED derived from the t(14;18) Mbr and mcr. The size of these fragments is usually between 150 and 300 bp and may, in a rare case, be up to 450 bp. Figure 1, panel R (red), contains the fragments of the molecular weight marker, labeled with ROX.

The multicolor multiplex PCR assay allows for the rapid screening of clinical samples for B- and T-cell clonality and for the most commonly occurring genetic changes in non-Hodgkin lymphoma, t(11;14) and t(14;18). The assay was applicable to fresh frozen and formalin-fixed paraffin-embedded tissue as well as to samples of blood and to bone marrow aspirates.

Materials and Methods

Cases/Tissues

The tissue probes used in this study were derived from surgical specimens obtained for diagnostic purposes. Tissues were routinely fixed in buffered formalin (4%) for at least 18 hours. They were then embedded in paraffin and stained according to standard methods. The diagnosis of malignant lymphoma was made according to the REAL classification and enforced by additional immuno-

histochemistry using a panel of monoclonal antibodies against line-specific antigens including CD3, CD4, CD8, TIA, CD56, and CD57 for T lymphocytes; and CD20, CD10, CD23, CD75, and CD79a for B lymphocytes. Additional markers were *bcl2* and *bcl6* for follicular non-Hodgkin lymphoma and cyclin D1 for mantle cell lymphoma. Molecular diagnosis was performed by use of conventional methods as follows: IgH rearrangements were analyzed by singleplex PCR for the IgH FR1 and FR3 regions, detection of the t(11;14) MTC and the t(14;18) Mbr and mcr was performed by nested PCR. Semi-nested multiplex PCR was used to analyze TCR- γ rearrangements. Electrophoresis was performed on 4% agarose gels. The results were confirmed by DNA sequence analysis of the PCR fragments derived from translocations and clonal rearrangements.

From these cases, 35 T-cell lymphomas with clonal TCR- γ rearrangements and 54 B-cell-lymphomas with proven clonality, including 11 mantle cell lymphomas bearing the t(11;14) and 25 follicular lymphomas bearing the t(14;18) were chosen to test the specificity of the multicolor multiplex PCR. Negative controls included 11 samples of reactive lymphadenitis. In addition, 12 samples of formalin-fixed, paraffin-embedded tissue derived from a European proficiency test, which had been diagnosed correctly by our conventional PCR methods, were tested. Cultured cells of the cell lines Jurkat, DOHH2, and REC-1 were used as positive controls.

DNA Extraction

Formalin-fixed and paraffin-embedded samples were deparaffinized according to standard methods. Lymphocytes were isolated from blood and bone marrow aspirates using Ficoll gradients (Histopaque-1077, Sigma, St. Louis, MO). DNA was extracted by use of the QIAamp Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. If only small amounts of tissue were available (eg, tissue scratched off glass slides), carrier RNA (artificial polyC RNA; Promega) was added before DNA precipitation. Whenever possible, DNA extractions were performed in duplicate. To estimate DNA yields, OD at 260 nm was measured or gel electrophoresis was performed using 0.8% agarose gels stained with ethidium bromide.

Primer Design and Testing

The primers were newly designed by us using the GeneWorks software (Intelligenetics), and optimized for the multiplex PCR. DNA sequences used for primer design were obtained from the EMBL database. EMBL accession numbers of the sequences used are as follows: TCR- γ locus, AF 159056; IgH locus, V genes, AB019437, AB019438, AB019439, AB019440, and AB019441; J genes, J00256. *Bcl-1* MTC (X74150), *BCL-2* Mbr (M13994), and *BCL2* mcr sequences were published by Ngan and colleagues.³⁵ The GCG (Genetics Computer Group Inc.) software was used. Primer pairs of each diagnostic test were extensively tested by monoplex and

partial multiplex PCR using clinical samples and the respective positive and negative controls before they were included into the multiplex PCR primer mix. As the quality of the primers is crucial to obtain clear-cut results, each primer was examined by capillary electrophoresis and laser-induced fluorescence detection, using a ABI 310 Genetic Analyzer (Applied Biosystems) before use. Samples contained 0.1 pmol of each primer, 1 μ l of the molecular weight marker ROX 500 (Applied Biosystems) and 25 μ l of deionized formamide (Applied Biosystems). Primers should show one clear peak. The peak area should be $\sim$ 70,000 fluorescence units, indicating efficient labeling with fluorophores. A stock solution was then prepared containing the primers listed in Table 1 according to the concentrations shown in Table 1 in pmol per reaction. The primer mix stock solutions were stored in aliquots at -20°C to avoid repeated freezing and thawing. Primers labeled with fluorophores were protected from light.

T- and B-Cell Multicolor Multiplex PCR

The reaction mix contained standard PCR buffer (Applied Biosystems), 0.2 mmol/L each dATP, dGTP, dCTP, and dTTP, the primer mix (Table 1) at the appropriate concentrations, and 10 U AmpliTaq Gold DNA polymerase per reaction. DNA (100 to 300 ng) was added and the final reaction volume was 25 μ l. PCR parameters were as follows: 95°C for 11 minutes; 36 cycles at 95°C for 40 seconds, 55°C for 40 seconds, 72°C for 60 seconds, and a final step of 20 minutes at 72°C to complete elongation and dA addition.

Automated HRFA and GeneScan Analysis

PCR products were analyzed by capillary electrophoresis and laser-induced fluorescence detection, using a ABI 310 Genetic Analyzer (Applied Biosystems). Procedures were according to the manufacturer's recommendations. Each sample contained 1.5 μ l of the PCR sample, 1 μ l of the molecular weight marker ROX 500 (Applied Biosystems), and 25 μ l of deionized formamide (Applied Biosystems). Samples were denatured at 95°C for 2 minutes and immediately chilled on ice. The gel used for CE was POP-4 polymer solution (Applied Biosystems). Results were visualized using the GeneScan analysis software (Applied Biosystems).

TCR- γ -Specific Multicolor Multiplex PCR

The multiplex PCR for the verification of TCR- γ fragments was performed essentially as described above, using the primer mix listed in Table 2, except that the amount of AmpliTaq Gold DNA polymerase was reduced to 5 U per reaction. Primer concentrations were according to Table 2.

DNA Sequence Analysis

In some cases, PCR fragments derived from chromosomal translocations and clonal IgH rearrangements

Table 1. Primer Mix of the B- and T-Cell Multicolor Multiplex PCR

Primer name	Primer sequences	Label	pmol per reaction
IgH V FR1			
FR1-A/B	GGTGCAGCTGGTGSAGTC	5-FAM (blue)	5
FR1-C	GGTGCAGCTGCAGGAGTC	5-FAM (blue)	2
FR1-V4	GACCCTCTCCCTCACCTGYG	5-FAM (blue)	2
FR1-V-1/7	CCTCAGTGAAGGTYCCTGC	5-FAM (blue)	2
FR1-V2	ACCTTGARGGAGTCTGGTCC	5-FAM (blue)	2
IgH V FR3			
FR3A	GAGGACACGGCTGTGTTACTGT	5-FAM (blue)	1.5
FR3B	GAGGACACGGCCGTGTTACTGT	5-FAM (blue)	1.5
t(11:14)			
BCL1-MTC	GAGCATAATTGCTGCACTGC	6-JOE (green)	2
t(14:18)			
BCL2-Mbr	AGATGGCGAATGACCAGC	NED (yellow)	2
BCL2-mcr	TCTTGCAAGGCTCTTAAGCAG	NED (yellow)	2
IgH J			
JOIN A	CCTGAGGAGACGGTGACC	None	8
JOIN B	CCTGAAGAGACGGTGACCA	None	2
JOIN C	CCTGAGGAGACAGTGACCAG	None	2
TCR- γ V			
V1cons.n	CCCACAGCRTCTTCTGTACTATG	5-FAM (blue)	2
V1S2 n	CCCACAGCGTCTTCAGTACTATG	5-FAM (blue)	2
V1S7 n	CCCACAGCATCTTCCATACTATG	5-FAM (blue)	2
V5P n	TGGGATTCCATCCTGAAAGC	5-FAM (blue)	2
V2	CCTGGTGAAGTCATACAGTTCC	5-FAM (blue)	3
V3P/V4P	CACTGGTACYGGCAGAAACC	5-FAM (blue)	3
V6P	CACTGGTACCAGCAGAAACT	5-FAM (blue)	2
TCR- γ J			
J1/J2	ACMAGTGTTGTTCCACTGCC	None	5
JP1	CTATGAGCTTAGTCCCTTCAGC	None	3
JP2	CTATGAGCCTAGTCCCTTTTGC	None	3
JP	ATAAGCTTTGTTCCGGGACC	None	3
β -Globin			
β -Globin1	GCCATCACTAAAGGCACCGAGC	6-JOE (green)	1.5
REV β -Globin2	ATGGTGACCTGACTCCTGAGG	None	1.5

The fluorescence labels are indicated. Primer concentrations are shown in pmol of primer per reaction. Symbols for mixed bases are as follows: R, A/G; Y, C/T; M, A/C; S, G/C. The designation of the TCR- γ primers corresponds to the nomenclature of the TCR- γ variable gene subgroups or families also used in the literature as follows: V1, V γ -I; V2, V γ -II (V9); V3, V γ -III (V10); V4, V γ -IV (V11); V5P, VA; V6P, VB. V1cons. = consensus primer for 6 of the 8 V γ -I genes, V1S2 = V γ -I gene 2, V1S7 = V γ -I gene 7.

could be sequenced directly from the multiplex reaction mix. Clonal TCR- γ rearrangements required the verification step to determine the family-specific primer to be used as a sequencing primer. However, results were greatly improved, if an additional monoplex PCR was performed with unlabeled primers of the corresponding test. In either case, 0.5 to 1 μ l of the PCR reaction mix were used without further purification to provide the DNA template. The corresponding unlabeled forward primers were used as sequencing primers. DNA sequencing was performed using the dRhodamine or Big Dye Terminator Kits (Applied Biosystems) according to the protocols provided by the manufacturer. Sequence analysis was performed on an ABI 310 Genetic Analyzer using the sequence analysis software (Applied Biosystems). Procedures were according to the manufacturer's recommendations.

Results

Primer pairs of each diagnostic test were extensively tested by monoplex and partial multiplex PCR using clinical samples and the respective positive and negative

controls before they were included into the multiplex PCR primer mix (data not shown). Results obtained from control cells and tissue by multicolor multiplex PCR and automated HRFA, using the GeneScan analysis software, are shown in Figure 2. Multiple signals in the expected size range and color panel (compare Figure 1) are created by polyclonal populations of B and T cells, as present in reactive lymphoproliferation and in thymus tissue (Figure 2A, panel B). Peaks derived from IgH FR3-J fragments appear in a range between 70 and 140 bp, those from IgH FR1-J fragments between 330 and 400 bp. Because of the short N regions present in TCR- γ rearrangements, the PCR fragments derived thereof are within a smaller size range, between 200 and 250 bp, with the bulk of fragments present in the size range between 210 and 230 bp. In contrast to the polyclonal pattern shown in Figure 2A, the clonal TCR- γ rearrangements of the T-cell line Jurkat, which has both its TCR- γ loci rearranged, are visible as two clear peaks at 209 and 245 bp (Figure 2B, panel B). The cell line REC-1, derived from a mantle cell lymphoma,¹³ bears a t(11;14). This results in amplification of a 229-bp fragment labeled with 6-JOE (Figure 2C, panel G). REC-1 cells have a rear-

Table 2. Primer Mix of TCR- γ -Specific Multicolor Multiplex PCR

Primer name	Primer sequences	Label	pmol per reaction
TCR- γ V			
V1-cons.	ATCCACTGGTACCTACACCAGG	6-JOE (green)	2
V1S5P	ATCCACTGGTACCTACAGCAGG	6-JOE (green)	2
V1S1P/7	ATCCACTGGTACCTGCACCAGG	6-JOE (green)	2
V1S6	ATCCACTGGTACCTACGCCAGG	6-JOE (green)	2
V5P	ACCCACTGGTACCAGCAGAAGG	6-JOE (green)	2
V2 n	CCATTTCATATGACGGCACTG	NED (yellow)	3
V3P n	ACAAGCAACAAAGTGGAGGC	6-FAM (blue)	3
V4P n	GACAATCTCTGCTCAAGATTGC	6-FAM (blue)	3
V6P n	CCCAGTTCGAGTTTGCTTAGG	6-FAM (blue)	2
TCR- γ J			
J1/J2	ACMAGTGTGTTCCTACTGCC	none	5
JP1	CTATGAGCTTAGTCCCTTCAGC	none	3
JP2	CTATGAGCCTAGTCCCTTTTGC	none	3
JP	ATAAGCTTTGTTCCGGGACC	none	3
Interpretation of Results*			Difference in fragment length
V1 fragments			+ 34 bp
V5P fragments			+ 78 bp
V2 fragments			-28 bp
V3P fragments			-89 bp
V4P fragments			-55 bp
V6P fragments			-65 bp

The fluorescence labels of the different TCR- γ V gene primers are indicated. Primer concentrations are shown in pmol of primer per reaction. The symbol for mixed bases is as follows: M = A/C. The designation of the TCR- γ primers corresponds to the nomenclature of the TCR- γ variable gene subgroups or families also used in the literature as follows: V1, V γ -I; V2, V γ -II (V9); V3, V γ -III (V10); V4, V γ -IV (V11); V5P, VA; V6P, VB; V1cons., consensus primer for 4 of the 8 V γ -I genes; V1S5P, V γ -I pseudogene 5; V1S1P/7, V γ -I pseudogene 1 and gene 7; V1S6, V γ -I gene 6.

*The length of a given PCR fragment obtained in the B- and T-cell multicolor multiplex PCR is compared to the length of the fragment obtained in the TCR- γ -specific multicolor multiplex PCR. The result is confirmed, if the difference in fragment length confirms to the subtype-specific value. The values listed above may vary by a few base pairs because of the denaturing power of the gel. The subtypes are determined by the color of the fluorescence label.

ranged IgH locus on the other allele, as shown by amplification of both a clonal IgH FR3-J and a FR1-J fragment. (Figure 2C, panel B). The cell line DOHH2, derived from a follicular lymphoma,³⁶ harbors a t(14;18) with its bcl-2 breakpoint in the Mbr region, as visualized by the signal at 243 bp in Figure 2D, panel Y. DOHH2 cells also have a rearranged IgH locus with V sequences detected by both the FR3 and the FR1 primers (Figure 2D, panel B). The amplification of the IgH FR3-J fragment is less efficient than that of IgH FR3-J fragment, indicating that point mutations in the primer-binding sequences are present in the FR3 region. A peak at 352 bp in panel G is always present and shows amplification of the control gene β -globin. Samples lacking DNA were always included as negative controls and did not show any signals (data not shown).

Serial dilutions of DNA from the control cell lines with DNA of the sample of reactive lymphoproliferation shown in Figure 2A were performed to test the sensitivity of the assay. Clonal IgH rearrangements could clearly be distinguished from the polyclonal background with 5% of REC1 or DOHH2 DNA. The ratio between the peak height of the clonal IgH rearrangement and the highest peaks of the polyclonal background was still 2:1. The same ratio between the peak heights from the clonal TCR- γ rearrangements was obtained with 10% DNA from Jurkat cells present in the polyclonal background. Using standard conditions, the t(11;14) and the t(14;18) fragments were detectable in the presence of 1% of REC1 or

DOHH2 DNA, respectively, corresponding to 1 ng of DNA or 150 cells.

All results shown below were obtained from formalin-fixed, paraffin-embedded tissue of clinical samples of T- and B-cell lymphomas. In clinical samples, nonneoplastic B and T cells are usually present together with the clonal cells of the lymphoma. The signals from the polyclonal IgH and TCR- γ rearrangements are often below detection, if the majority of the probe consists of clonal B cells, or visible as small peaks from which a clonal IgH fragment can clearly be distinguished (Figure 3). In the majority of B-cell lymphomas, signals from clonal IgH rearrangements from both IgH FR3-J and FR1-J fragments are obtained (Figure 3A). This provides additional security with respect to the result. In a few variable IgH genes, the primers used in the multiplex PCR correspond either to sequences of the FR3 region or to those of the FR1 region, but not to both. More often, this will be the case in samples of lymphomas that are subject to somatic hypermutation, eg, follicular lymphomas. Accordingly, only one of the two IgH regions analyzed will show the signal of a clonal IgH rearrangement. Examples are shown in Figure 3B (FR3) and Figure 3C (FR1). The FR1 region of seven of the variable IgH genes can be detected by two different primers designed for the FR1 region. Clonal IgH rearrangements involving these genes may show two peaks in the FR1-specific size range. Thus, clonal fragments from both the FR1 and the FR3 regions or from either one only may be detected, depending on V gene usage, and,

Figure 2. Analysis of control cells by multicolor multiplex PCR, automated HFRA, and GeneScan analysis. Fragments are aligned by size, which is indicated above the panels. **A:** Polyclonal populations of B and T cells present in a sample of lymphadenopathy. **Panel B:** Multiple IgH V FR3-J and V FR1-J fragments from polyclonal B cells, and multiple fragments derived from polyclonal TCR- γ rearrangements; **panel G,** peak of the β -globin fragment at 352 bp. **B:** The T-cell line Jurkat. **Panel B:** Fragments from the two clonal TCR- γ rearrangements at 209 bp (V1-J1/J2) and 244 bp (V4P-J1/J2); **panel G,** β -globin at 352 bp. **C:** REC-1 cells, derived from a mantle cell lymphoma. **Panel B:** V FR3-J (103 bp) and V FR3-J (365 bp) fragments of a clonal IgH rearrangement; **panel G,** peak of the t(11;14) fragment at 229 bp, β -globin at 352 bp. **D:** DoHH2 cells, derived from a follicular lymphoma. **Panel B:** V FR3-J (93 bp) and V FR1-J (364 bp) fragments of a clonal IgH rearrangement; **panel G,** β -globin at 352 bp; **panel Y,** peak of the t(14;18) Mbr fragment at 242 bp.

in a rare case, two FR1 fragments of different lengths may appear in the FR1-specific size range. Visualization of results can be further improved, if required, by focusing on the size range of the individual tests. In Figure 4, the focus is on the IgH FR3-J-specific size range. A polyclonal pattern of IgH rearrangements derived from non-neoplastic B cells is present together with a clonal IgH rearrangement in the sample of a B-cell lymphoma. Interestingly, the signals obtained from the polyclonal B-cell populations are spaced by 3 bp, and the fragment lengths show, that in the majority of IgH rearrangements the reading frame with respect to the join gene is maintained. The specificity of the PCR fragments derived from clonal IgH rearrangements was determined by DNA sequencing of the individual V-N-J junctions of 22 cases. In the majority of the cases, the reading frame of the join gene was not altered by the insertion of N sequences.

Multiplex PCR was performed on 11 cases of mantle cell lymphoma harboring a t(11;14) and on 25 cases of

follicular lymphoma harboring a t(14;18), which had previously been analyzed by nested PCR. Strong signals from the PCR fragments of the appropriate lengths were obtained after a single round of multiplex PCR in all cases. Amplification was highly specific, as we did not observe any false-positive results in a large number of samples analyzed. Cells of mantle cell lymphomas and follicular lymphomas may have their IgH locus rearranged on the other allele. Clonal IgH rearrangements could frequently be demonstrated together with the specific chromosomal translocation. Examples are shown in Figure 5. A clonal IgH rearrangement (Figure 5A, panel B) and a t(11;14) (Figure 5A, panel G) are present in the cells of a mantle cell lymphoma. Results obtained from a follicular lymphoma show a t(14;18) (Figure 5B, panel Y) and an IgH rearrangement on the other allele of chromosome 14, whose sequences correspond to the IgH-FR1 but not to the IgH-FR3 primers (Figure 5B, panel B). Polyclonal T cells are present in the same sample. A

Figure 3. Analysis of clinical samples of B-cell lymphomas by multicolor multiplex PCR, automated HRFA, and GeneScan analysis. Fragments are aligned by size, which is indicated above the panels. **A:** Sample of a B-cell lymphoma with signals of a clonal IgH rearrangement from both V FR3-J (104 bp) and V FR1-J (369 bp) fragments and low polyclonal background of nonneoplastic B and T cells. **B:** B-cell lymphoma with signal of a clonal IgH rearrangement from a V FR3-J (125 bp) fragment, and polyclonal V FR1-J and TCR- γ fragments. **C:** B-cell lymphoma with signal of a clonal IgH rearrangement from a V FR1-J (374 bp) fragment, and polyclonal V FR3-J and TCR- γ fragments.

Figure 4. Focus on the IgH FR3 region-specific size range. Signals of a clonal IgH rearrangement (133 bp) and nonneoplastic B cells present in the same sample of a B-cell lymphoma. The majority of the IgH V-J fragments differ in length by a multiple of 3 bp.

follicular lymphoma bearing a t(14;18) with its bcl-2 breakpoint in the mcr is shown in Figure 5C, panel Y. In this sample, the IgH locus is not amplified. Small amounts of polyclonal T cells are visible, signals from polyclonal B cells are almost below detection (Figure 5C, panel B). DNA sequence analysis of the PCR fragments derived from 11 t(11;14) and 25 t(14;18) was performed. The individual breakpoints of the t(11;14) were within a range of 76 bp in the MTC (Figure 6). The bcl-2 breakpoints of 25 follicular lymphomas were clustered within a 122-bp region of the Mbr (Figure 6). One t(14;18) had its bcl-2 breakpoint in the mcr.

T cells may have one or both of their TCR- γ loci rearranged. Accordingly, clinical samples of T-cell lymphomas usually show one or two clear peaks when analyzed by the multiplex PCR assay. An example is shown in Figure 7A. Considerable amounts of nonneoplastic T and B cells may be present in some samples of T-cell lymphomas and cause a high background of polyclonal signals. A clonal TCR- γ rearrangement present within the bulk of polyclonal fragments can still clearly be distinguished, as its peak height is well above that of the polyclonal background (Figure 7B). From the sample shown in Figure 7B, only partially degraded DNA was obtained, as is frequently the case if formalin-fixed, paraffin-embedded tissue is used. The control gene β -globin is still amplified. Likewise, clonal IgH FR1-J fragments would be amplified, but the weak signals of the polyclonal IgH FR1-J fragments remain below detection. If results seem ambiguous because of high polyclonal background, an additional, independent multicolor multiplex PCR, specifically developed for the analysis of TCR- γ rearrangements, can be performed. This allows confirming of the results and to determine the subtype of the V gene present in a clonal TCR- γ rearrangement. This PCR assay separates fragments derived from TCR- γ rearrangements by different fluorescence colors, according

Figure 5. Analysis of clinical samples of mantle cell and follicular lymphoma by multicolor multiplex PCR, automated HRFA, and GeneScan analysis. Fragments are aligned by data points, which are indicated above the panels. Fragment lengths are listed below the panel. **A:** A clonal IgH rearrangement (**panel B**) with both V FR3-J (96 bp) and V FR1-J (361 bp) fragments, and t(11;14) (**panel G**, 231 bp) are present in a mantle cell lymphoma. **B:** A follicular lymphoma bearing a t(14;18)Mbr (**panel Y**, 242 bp). The IgH FR1-J fragment from a clonal IgH rearrangement (**panel B**, 363 bp) is amplified. Polyclonal signals from nonneoplastic T cells are present in the same sample (**panel B**). **C:** A follicular lymphoma bearing a t(14;18) mcr (**panel Y**, 225 bp) and polyclonal signals from nonneoplastic B and T cells present in the same sample (**panel B**).

to the V gene subtype present in the rearrangement: V1 (V γ -I) and V5P (VA) genes appear in panel B (blue); V2 (V γ -II) genes in panel Y (yellow); and V3P (V γ -III), V4P (V γ -IV), and V6P (VB) genes in panel G (green). In addition, a different set of V primers is used (Table 2), resulting in PCR fragments that differ in their length from those obtained in the first multiplex PCR. These differences in fragment length are specific for each of the V gene subtypes. Expected differences in fragment lengths are listed in Table 2. These can vary by a few bp, because of the denaturing power of the gel. An example is shown in Figure 7, C and D, respectively. Two peaks of 212-bp and 241-bp length in Figure 7C, panel B, show the presence of two clonal TCR- γ rearrangements (Figure 7C). The TCR- γ -specific multiplex PCR clearly confirms the result: A fragment of 149 bp present in Figure 7, panel B, shows a clonal TCR- γ rearrangement containing a V3P gene (difference in fragment length, 90 bp). The 245-bp fragment present in Figure 7, panel G, shows a second clonal TCR- γ rearrangement with a V1 gene (difference in fragment length, 33 bp).

Discussion

The suggested multicolor multiplex PCR assay detects clonal B- and T-cell rearrangements and the chromosomal translocations t(14;18) and t(11;14) with high specificity. Ninety-seven samples of B- and T-cell lymphomas, bearing rearrangements and/or translocations, as previously determined by conventional methods and confirmed by DNA sequence analysis, yielded the same results when analyzed by multicolor multiplex PCR, and we did not detect any false-positive results in a large number of samples tested. In addition, the assay provides semiquantitative information with respect to B- and T-cell populations present in the same sample and monitors the quality of the extracted DNA and the efficiency of the PCR reaction by amplification of a housekeeping gene. DNA obtained from formalin-fixed, paraffin-embedded tissue is frequently partially degraded, with the majority of DNA fragments below 400 bp. Accordingly, amplification of fragments larger than 400 bp is not possible by use of standard PCR parameters. In these cases, PCR analysis of the IgH FR3-J region, the TCR- γ rearrangements (Figure 7B) and the vast majority of the t(14;18) and the t(11;14) is still possible, whereas IgH FR1-J fragments and larger fragments from the translocations (which are very rare) remain below detection. The 352-bp β -globin control fragment still allows monitoring of successful PCR in these cases. A small peak height of the β -globin fragment indicates that either the DNA concentration was too low, or the DNA is severely degraded.

Thus, the status of a clinical sample with respect to B- and T-cell populations and to the most frequent chromosomal translocations occurring in B-cell lymphomas can be evaluated simultaneously in a single reaction. This provides an enormous time and labor savings when compared to current methods that need several separate PCRs for each of these diagnostic tests. In addition, using capillary electrophoresis and laser-induced fluo-

Breakpoints BCL-2 MBR

* * * * *

CCTGCCCTCCTTCGCGGGGGCTTTCTCATGGCTGCTCTTCA GGGTCTTCCTGAAATGCAGTGGTGCTTACGCTCCACCAAGAAAG CAGGAAACCTGTGGTATGAAGCCAGACCTCCCGCGC

CCTG * ACCAGGATCCAAAGAT TCGACTTCTGGGGCC
 CCTGCCCTCCTTC * AGGCCTAGAAGGCCAT ACTACTACTACTACGGTATGGAAGTCTGGGGCC
 CCTGCCCTCCTTC * ATGCC CC
 CCTGCCCTCCTTC * AGGCCTATCCTTATAACCTCATAGGCCGATT ACTACTACTACTACTACATGGAAGTCTGGGGCC
 CCTGCCCTCCTTC * GGCACATGTGGGGGACCGCAAAAGCGAGGGGGTTTTC CTACTACTACTACTACATGGAAGTCTGGGGCC
 CCTGCCCTCCTTC * GGAGTAAGGGGGGAACCT TACTACTACGGTATGGAAGTCTGGGGCC
 CCTGCCCTCCTTCGCG * TTCAGTGTAC GGGGGC

GGGTC * CCGGCTACGGAGTCTTGAA ACTGGTTGACCCCTGGGGCC
 GGGTCTTCCTGAAATGCAG * TGGTCTTACGCTCCACCAAGAAC CTACGGTATGGAAGTCTGGGGCC
 GGGTCTTCCTGAAATGCAGTGGT * GCTCCCTGGTTCCTCCGAA TACTACTACTACTACGGTATGGAAGTCTGGGGCC
 GGGTCTTCCTGAAATGCAGTGGT * ATTGCTC CTACTACTACGGTATGGAAGTCTGGGGCC
 GGGTCTTCCTGAAATGCAGTGGT * GCGAACTTAACTGGAAGTACTACATACCCGGG TACTACTACTACTACATGGAAGTCTGGGGCC
 GGGTCTTCCTGAAATGCAGTGGT * CCGTGGTTCCTCCGAA ACTACTACTACTACGGTATGGAAGTCTGGGGCC
 GGGTCTTCCTGAAATGCAGTGGT * TAGGAAGACCG ACTACTACTACTACGGTATGGAAGTCTGGGGCC
 GNGTCTTCCTGAAATGCAGTGGT * AACGAAAGGACCTCCCTTTGGGTTTCCCTTACGG TATGGAAGTCTGGGGCC
 GGGTCTTCCTGAAATGCAGTGGT * TAACTCTCCCTGGGG CTACTACTACTACGGTATGGAAGTCTGGGGCC
 GGGTCTTCCTGAAATGCAGTGGT * GGAAGACCGAAG ACTACTACTACTACGGTATGGAAGTCTGGGGCC
 GGGTCTTCCTGAAATGCAGTGGT * GACCTTTTGTAGTCCCTACCCCATGTAGTATTCCGACTA CTGGGGCC

CAGGA * TAGATCGCCAGGTGGTGTGCTGCGTGGCAGTCTACGGTCTACTTACAAGTGGTTCGACCG CTGGGGCC
 CAGGA * TTTT ACTACTACTACTACGGTATGGAAGTCTGGGGCC
 CAGGAAACCTGTGGTATGAAGCCAGACCTCC * TTTTCACTC TACGGTATGGAAGTCTGGGGCC
 CAGGAAACCTGTGGTATGAAGCCAGACCTCC * AAACTATTGGTTGG ACTACTACGGTATGGAAGTCTGGGGCC
 CAGGAAACCTGTGGTATGAAGCCAGACCTCC * - TACTACTACTACGGTATGGAAGTCTGGGGCC
 CAGGAAACCTGTGGTATGAAGCCAGACCTCC * ATTTGGTCCAAA CTACTACTACATGGAAGTCTGGGGCC
 CAGGAAACCTGTGGTATGAAGCCAGACCTCCCGC * CAAAGCCTGAACCTACTTTG ACTACTGGGGCC

Breakpoints BCL-1 MTC

* * * * *

GATGAGATTAACTGGGTCTTCTCGTGGTTTGAACGCAAGAGCTCCCTGAACACCTGGCGCWGCCATTGGCGTGAA

GATGAGA * ACTCAGATTCCAAACATATT ACTACTACTACTACGGTATGGAAGTCTGGGGCC
 GATGAGA * AMAMCCGGCCGGGACCTAG ACTTTGACTACTGGGGCC
 GATGAGATT * CGCTGTTCTCTGCTTCTGTSTGCTTT GTTCGACTTCTGGGGCC
 GATGAGATTAACT * TCCT TTCGGCCCTGGGGCC
 GATGAGATTAACTGCG * GGTCCAAGGA TTGACTACTGGGGCC
 GATGAGATTAACTGCGTCTTCTTCTGTTTGAACGCAAGAGCTCCCT * ATACCCCGGGAGGT ACTACGGTATGGAAGTCTGGGGCC
 GATGAGATTAACTGCGTCTTCTTCTGTTTGAACGCAAGAGCTCCCT * CGAGGGAGTC ACTGGTTCGACCCCTGGGGCC
 GATGAGATTAACTGCGTCTTCTTCTGTTTGAACGCAAGAGCTCCCTGGAACACCTGGCGCTGCCATTG * ACCCTAAATAT ACTACTACTACTACATGGAAGTCTGGGGCC
 GATGAGATTAACTGCGTCTTCTTCTGTTTGAACGCAAGAGCTCCCTGGAACACCTGGCGCTGCCATTGGCGTGA * GATCCTCGTAGGGTGGGA GGCTACTGGGGCC
 GATGAGATTAACTGCGTCTTCTTCTGTTTGAACGCAAGAGCTCCCTGGAACACCTGGCGCTGCCATTGGCGTGA * ATTTAGGGTGGTGGTCCCAAT ACTACTTTGACTACTGGGGCC

Figure 6. Breakpoints in the bcl-2 region and sequences of t(14;18) junctions and breakpoints in the bcl-1 region and sequences of t(11;14) junctions. The breakpoints found in 25 cases of follicular lymphomas are indicated by asterisks. The sequences of the bcl-2-N-IgH J junctions are listed below. The breakpoints on chromosome 11 found in 11 cases of mantle cell lymphoma are indicated by asterisks. The sequences of the bcl-1-N-IgH J junctions are listed below.

rescence detection, hands-on time is further reduced. Routine screening for the most important molecular markers with respect to lymphoproliferative disorders is now feasible and may reveal, especially in atypical or polymorphous samples, information that would otherwise go undetected. In addition, lineage-inappropriate gene rearrangement becomes obvious. The simultaneous evaluation of B-cell clonality and the chromosomal translocations t(11;14) and t(14;18) is very useful, as the specific chromosomal translocations are demonstrable by PCR in only approximately half of the mantle cell- and follicular lymphomas. Mantle cell lymphomas frequently have a rearranged IgH locus on the other allele and do not show considerable somatic hypermutation of IgH V genes. Thus, in cases with undetectable t(11;14), a clonal IgH rearrangement can be demonstrated. In contrast, follicular lymphomas exhibit extremely high degrees of somatic hypermutation,³⁷ which might impair primer binding. Because two different IgH framework regions are analyzed in the assay, and the IgH FR3 primers are designed to tolerate point mutations, chances to show clonality despite of somatic hypermutation are greatly increased. Indeed we found that in a number of follicular

lymphomas apparently only one of the two IgH framework regions was affected by mutations and clonality could still be shown by amplification of the other.

The use of fluorescence-labeled primers and automated HRFA greatly improves sensitivity, separation, and visualization of PCR fragments when compared to conventional methods, eg, analysis by agarose or polyacrylamide gel electrophoresis. The higher sensitivity allows reducing of the number of PCR cycles, which is an asset in many respects. The excellent separation of the PCR fragments, which allows distinguishing of fragments differing in length by only 1 bp, is crucial for the analysis of IgH and, even more importantly, TCR- γ rearrangements. Using conventional methods, the polyclonal background caused by nonneoplastic T and B cells frequently present in clinical samples may obscure clonal rearrangements. Using HRFA, peaks from clonal rearrangements usually rise clearly above those derived from polyclonal background. The ratio between the peak height of a clonal rearrangement and the polyclonal background depends on the amount of reactive lymphocytes present in a sample together with the malignant clone. The ratio between the peak height of clonal IgH rearrangements and poly-

Figure 7. Analysis of clinical samples of T-cell lymphomas by multicolor multiplex PCR, automated HRFA, and GeneScan analysis. **A:** Two peaks in the TCR- γ -specific size range in **panel B** demonstrate the clonal TCR- γ rearrangements present in the sample of a T-cell lymphoma. **B:** TCR- γ rearrangement on one allele and background signals from polyclonal TCR- γ rearrangements of nonneoplastic T cells present in a peripheral T-cell lymphoma. Signals from polyclonal B cells present in the same sample are visible in the IgH-FR3-specific size range, but below detection in the IgH-FR1-specific size range, indicating partial degradation of the DNA sample. **C:** A T-cell clone with TCR- γ rearrangements on both alleles is present in a high background of nonneoplastic B and T cells in the sample of a peripheral T-cell lymphoma. The clonal TCR- γ rearrangements are visible as two peaks at 212 and 241 bp (**panel B**). Analysis of the same T-cell lymphoma by the TCR- γ -specific multicolor multiplex PCR is shown in **D**; a fragment of 149 bp present in **panel B** shows a clonal TCR- γ rearrangement containing a V3P gene (difference in fragment length, 90 bp). The 245 bp fragment present in **panel G** shows a second clonal TCR- γ rearrangement with a V1 gene (difference in fragment length, 33 bp).

clonal background was $>2:1$, if 5% of the cells were clonal. This ratio may be lower because of mutations in the primer-binding sequences of the clone, a problem encountered mainly in follicular lymphomas. An important control is the reproducibility of the clonal peak. Therefore, diagnostic tests should always be performed in duplicate. Because of their limited size range, TCR- γ rearrangements may have lower clonal:polyclonal ratios. We recommend performing the TCR- γ -specific multiplex PCR in all cases with a possible diagnosis of T-cell lymphoma, that do not show a ratio of at least 2:1. This assay further separates the fragments derived from TCR- γ rearrangements by different colors, and the results are confirmed by an independent assay. In all cases with a low clonal:polyclonal ratio, the small amount of clonal cells present in a larger polyclonal population should be interpreted with caution. Monoclonality is not a proof of malignancy and the diagnosis of malignant lymphoma

should only be made, if additional morphological criteria are met.

In samples with low numbers of B or T cells, eg, microdissected tissue, evaluation of clonality should be performed with caution. Pseudoclonal peaks of randomly amplified IgH or TCR- γ rearrangements can be produced, especially if the number of cycles is increased to compensate for the low amount of DNA present. These peaks are usually not reproducible.

We showed, that the multicolor multiplex PCR is a useful tool for rapid screening of clinical samples with at least 5 to 10% of clonal B or T cells or 1% of cells bearing a t(11;14) or a t(14;18) present in a polymorphous background. Using our standard conditions the sensitivity, with respect to the translocations, is limited by the small amount of DNA template, 1% corresponding to 1 ng of DNA, the equivalent of 150 cells. Applications requiring higher sensitivity, eg, screening for minimal residual dis-

ease, should be performed by monoplex PCR with primers specific for the malignant clone, using higher DNA and primer concentrations. High sensitivity together with high specificity can be achieved by using one primer that corresponds to the individual sequences of the V-N-J junction of the clone-specific rearrangement or translocation (VM and AR, unpublished results). This implies DNA sequence analysis of the PCR fragments, which can be performed using the rapid methods described here. In this study, the specificity and sensitivity of the four-color multiplex PCR was shown. To establish detection rates in a series of unselected lymphomas, larger studies will have to be performed. However, as detection rates will never be absolute, it should be kept in mind, that a polyclonal or negative result, respectively, does not exclude the presence of a malignant clone.

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