

ADVANCES IN DERMATOLOGY

Genotypic Analysis in Primary Cutaneous Lymphomas Using the Standardized Biomed-2 Polymerase Chain Reaction Protocols

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Abstract. The European Biomedicine and Health (BIOMED-2) Concerted Action Project BMH4-CT98-3936 has defined standardized protocols for polymerase chain reaction (PCR) amplification of different loci of the T-cell receptor (TCR) and immunoglobulin (Ig) genes with a view to achieving greater sensitivity and specificity in the assessment of clonality of lymphoid neoplasms. To assess T-cell clonality, analysis of TCR β gene and TCR δ rearrangements (useful in cases of T $\gamma\delta$ + cell neoplasms) is proposed alongside that of TCR γ . For analysis of B-cell clonality, along with the framework (FR) III segment of the IgH gene, other segments are studied (FRI, FRII) in addition to Ig λ and Ig κ genes or incomplete DJ rearrangements of the IgH gene and the κ deleting element. The results of the amplification are read using automatic reading systems (GeneScan) or using a heteroduplex system.

Key words: cutaneous lymphoma, gene rearrangement, clonality, polymerase chain reaction, BIOMED-2, GeneScan.

APLICACIÓN DE LOS PROTOCOLOS DE PCR BIOMED-2 EN EL ANÁLISIS GENOTÍPICO DE LOS LINFOMAS CUTÁNEOS PRIMARIOS

Resumen. El proyecto europeo BIOMED-2 Concerted Action BMH4-CT98-3936 describe protocolos estandarizados de amplificación por reacción en cadena de la polimerasa (PCR) de los diferentes *loci* de los genes del receptor de la célula T (TCR) e inmunoglobulinas (Ig) con el objetivo de obtener una mayor sensibilidad y especificidad en el estudio de clonalidad de las neoplasias linfoides. En el estudio de clonalidad T, además del TCR γ se propone el estudio adicional de los reordenamientos del gen TCR β y del *locus* TCR δ (útil en casos de neoplasias de células T $\gamma\delta$ +). En el estudio de clonalidad B, junto al segmento FRIII del gen IgH se diseña el estudio de otros segmentos (FRI, FRII), así como de los genes Ig λ e Ig κ o reordenamientos incompletos DJ del gen IgH y κ de (*kappa deleting element*). La lectura de los resultados de la amplificación se realiza mediante sistemas automatizados de lectura (GeneScan) o mediante el sistema heterodúplex.

Palabras clave: linfoma cutáneo, reordenamiento genético, clonalidad, reacción en cadena de la polimerasa, BIOMED-2, GeneScan.

Introduction

In recent years, molecular biology and pathology laboratories have introduced a set of molecular biology techniques based on polymerase chain reaction (PCR) into their diagnostic

procedures. The aim of these techniques is to demonstrate the presence of a dominant proliferation of a monoclonal T- or B-cell population in atypical cutaneous lymphoid infiltrates (genotyping).¹⁻³ The results of genotyping, when interpreted in the appropriate clinical and pathological context, represent an important diagnostic tool in lymphoid malignancies (lymphomas), particularly in those cases in which the morphologic findings and immunophenotyping are inconclusive.^{4,5}

PCR techniques can be used not only with fresh or frozen samples, but also with archival paraffin-embedded samples. The only requisite is that relatively small DNA fragments are conserved (>100-200 base pairs [bp]).¹ This is doubtlessly an advantage over other techniques, such as Southern blotting, which requires large DNA fragments (>1000 bp)

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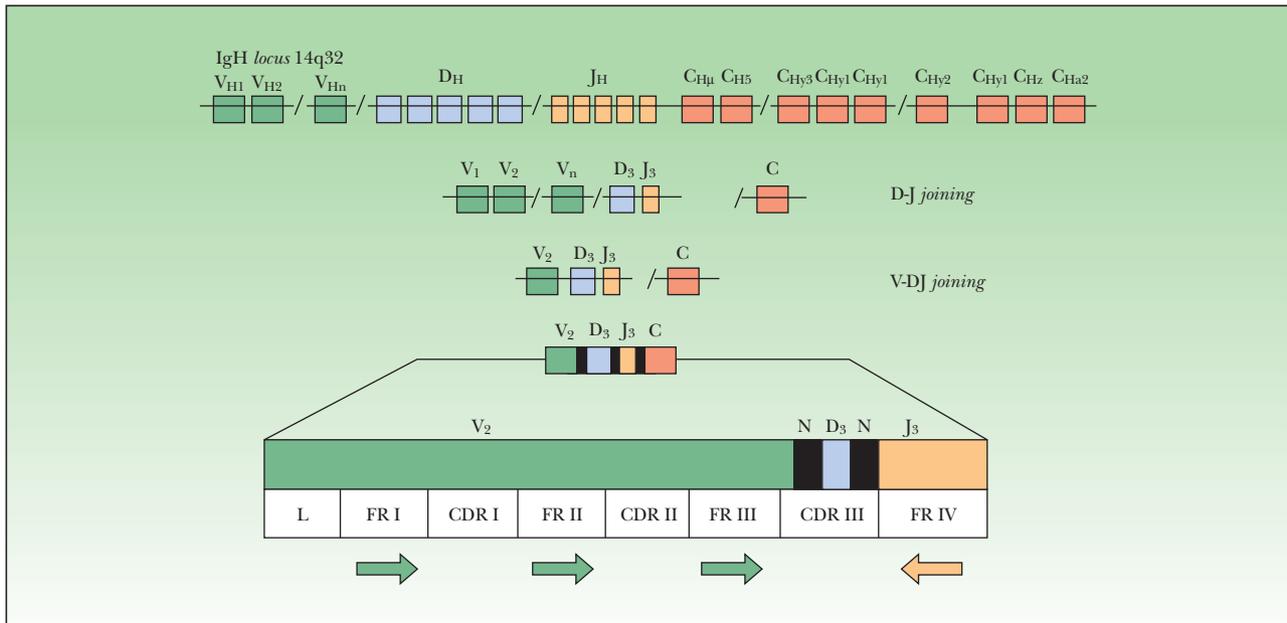


Figure 1. VDJ rearrangement of the IgH gene. The V regions of the IgH gene are formed from 3 hypervariable or complementary determining regions (CDR) I, CDRII, and CDRIII. These CDR segments alternate with 4 segments of little variability denoted framework regions (FR), where hybridization of the PCR primers occurs (arrows).

and which is therefore limited to the study of fresh or frozen material.^{6,7} PCR-based techniques avoid the need for exposure to radioactive agents, produce results more rapidly, and require less work in the laboratory. The differences in methodology between laboratories lie mainly in the choice of oligonucleotide primers and in systems for reading or analyzing the amplified PCR fragments.^{2,8-14}

The European Biomedicine and Health (BIOMED-2) Concerted Action Project BMH4-CT98-3936 was designed with a view to developing standardized PCR protocols for studying B- and T-cell clonality for diagnostic purposes in lymphoproliferative processes. It defines different protocols using multiple primers for different loci of T- and B-cell receptor genes in an attempt to increase the sensitivity and specificity of detection of T- and B-cell clonality.^{15,16}

T-Cell Receptor/Immunoglobulin H Gene Rearrangement

The B-cell receptor is an immunoglobulin (Ig)—specific to each cell—that is formed from 2 heavy chains (κ chains) and 2 light chains (λ chains). The T-cell receptor (TCR) is formed by joining an α subunit to a β subunit (α/β phenotype) or a γ subunit to a δ subunit (γ/δ phenotype). The genes that encode Ig and TCR proteins have different variable (V), diversity (D), and joining (J) segments that

undergo rearrangement during the initial phases of lymphoid cell differentiation.^{17,18}

This process of gene rearrangement is initiated by joining one of the possible D segments to one of the possible J segments. In the case of IgH, TCR β , and TCR δ genes, one of the V segments is subsequently joined (Figure 1). In the case of Ig λ , Ig κ , TCR α , and TCR γ genes, V and J segments are joined directly (Figure 2). This rearrangement follows a hierarchical order. During differentiation into a B cell, the IgH gene is rearranged first, followed by the Ig κ gene, which is subsequently deleted to form an Ig λ^+ cell. During differentiation into a T cell, first the TCR δ gene undergoes rearrangement, followed by the TCR γ gene, then the TCR β gene, with subsequent deletion of the TCR δ gene, and finally the TCR α gene is rearranged. The functional rearrangement of the Ig and TCR genes generates the expression of different receptors (Ig, TCR $\gamma\delta$, or TCR $\alpha\beta$) on the cell surface. These TCR $\gamma\delta$ or TCR $\alpha\beta$ configurations appear in the post-thymic T cells, with the TCR $\gamma\delta$ configuration corresponding to the minor fraction.¹⁸

The IgH gene (chromosome 14q32.3) has between 46 and 52 functional V_H segments depending on each individual's haplotype. These haplotypes can be grouped into 6 or 7 subgroups or families according to homology. In addition, there are 27 functional D_H segments grouped into 7 families (D_H1-7) and 6 functional J_H segments. The Ig κ gene (chromosome 2p11.2) contains multiple V κ segments

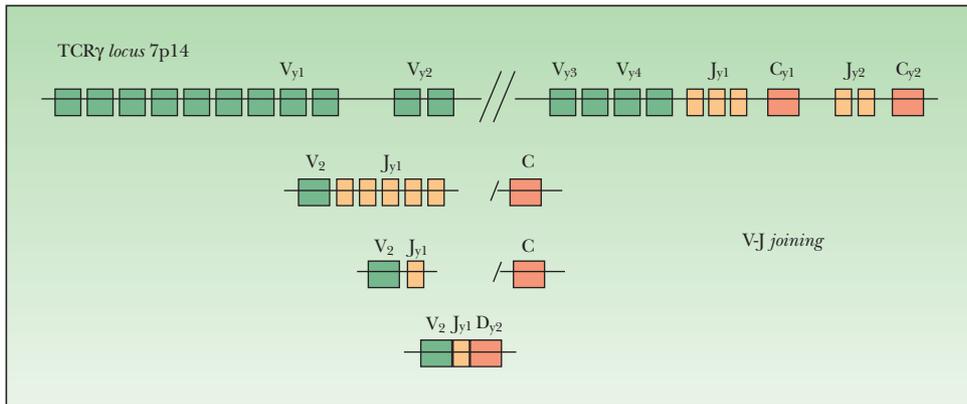


Figure 2. VJ rearrangement of the TCRγ gene.

grouped into 7 families and 5 functional J_H segments. The $Ig\lambda$ gene (chromosome 22q11.2) has 73 or 74 $V\lambda$ segments grouped into 11 families and 7 $J\lambda$ segments (4 functional: $J\lambda 1$, $J\lambda 2$, $J\lambda 3$, and $J\lambda 7$) that encode the 4 $Ig\lambda$ isotypes.^{17,19}

The TCRγ locus (chromosome 7p14) has 14 variable $V\gamma$ gene segments (10 functional or combinatorial); 5 $J\gamma$ joining segments belonging to 1 of 2 groups, $J\gamma 1$ ($J\gamma p 1$, $J\gamma p$, and $J\gamma 1$) and $J\gamma 2$ ($J\gamma p 2$ and $J\gamma 2$); and 2 constant regions $C\gamma 1$ and $C\gamma 2$. The $V\gamma$ segments are separated into 4 subgroups according to their homologous sequence: group I ($V\gamma 1-8$) has 5 functional segments and 4 pseudogenes; groups II, III, and IV are formed from single segments, $V\gamma 9$, $V\gamma 10$, and $V\gamma 11$, respectively. The TCRγ gene (chromosome 7q34) is formed by rearrangement of 1 of the 65 $V\beta$ segments (39-47 of which are functional and grouped according to homology into 23 families) with 1 of the $J\beta$ segments (2 families, $J\beta 1-2$).¹⁸

The combinatorial nature of V, D, and J segments gives rise to a repertoire of 2×10^6 possible combinations in the case of Ig, 3×10^6 possible rearrangements for TCRαβ, and 5×10^3 TCRγδ molecules. The diversity of these possible combinations is increased through insertion or deletion of nucleotides at segment joining regions, giving rise to more than 10^{12} possible combinations in the total repertoire of Ig and TCR molecules. Finally, in the case of B cells, the variability can also be increased through somatic hypermutations in the V segment of the IgH gene and the light chains of the mature B cells on their transit through the germinal center of lymphoid follicles, thereby further increasing the diversity and antigen specificity.¹⁷⁻¹⁹

Technical Aspects of the BIOMED-2 Project

DNA Quality Control

A crucial aspect in the application of the BIOMED-2 program is DNA quality control, which is particularly

relevant in the case of paraffin-embedded samples. It is recommended to run a PCR to amplify control genes with 5 primer pairs for amplification of 100, 200, 300, 400, and 600 bp fragments. This gives an indication of the quality of the DNA in the sample analyzed. All PCR fragments in the BIOMED-2 project have fewer than 600 bp (Figure 3). PCR should be done with 2 different amounts of DNA to exclude the effect of PCR inhibitors (Table 1).¹⁵

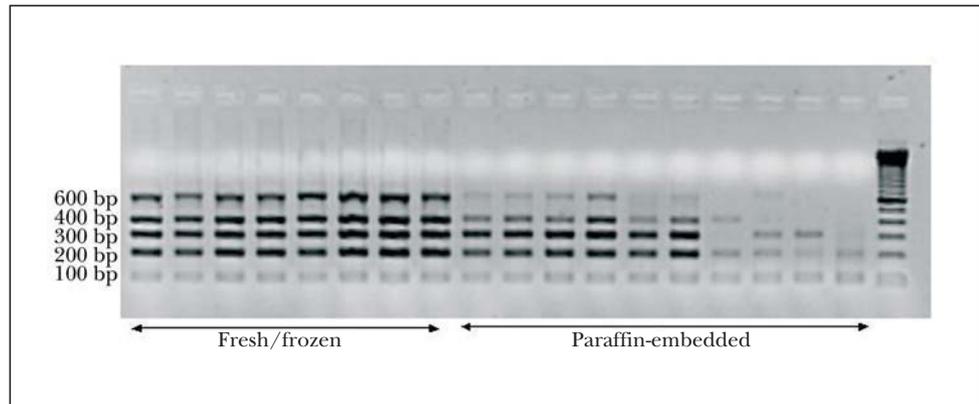
Fresh or frozen tissue is the best for obtaining good quality DNA; however, in routine practice, most samples studied will be formalin-fixed and embedded in paraffin. The DNA obtained from such samples is usually of poor quality, particularly when the sample is more than 2 years old. The fixation procedure used and the quality of the tissue studied are also important factors,²⁰ and so it is essential to run a control of DNA quality before amplification. If, in the quality control procedure, the DNA obtained is not longer than the gene or segment amplified, the results obtained are usually meaningless. It is also advisable to perform histologic control at the same time as obtaining tissue for DNA extraction to ensure that the sample studied is representative.

Analysis of PCR Fragments

Recently, automated reading systems have been developed for amplifying PCR fragments; these afford greater discriminatory power than traditional electrophoresis-gel-based reading systems.²¹⁻²⁸ The BIOMED-2 project recommends reading PCR amplified products by capillary electrophoresis with fluorochrome-labeled primers (GeneScan) or using polyacrylamide gels for heteroduplex analysis.²⁹⁻³¹

As all the cells of a lymphoid malignancy originate from a malignant clone, their DNA will have undergone the same rearrangement, and so on studying VDJ rearrangement by PCR, 1 or 2 PCR products will be obtained (rearrangement of 1 or 2 alleles). In contrast, if the DNA

Figure 3. Polymerase chain reaction (PCR) quality control of DNA. Quality control of DNA obtained from fresh/frozen samples and paraffin-embedded samples. The largest band indicates the maximum amplifiable size of a DNA fragment using PCR.



is derived from a polyclonal population, many VDJ rearrangements will be present. Visualizing the PCR product can be done by acrylamide gel electrophoresis or capillary electrophoresis. With gel electrophoresis, many discrete bands will be seen in the case of a polyclonal population or 1 or 2 bands in the case of a monoclonal population. Capillary electrophoresis reveals multiple peaks with a normal distribution of molecular weights for polyclonal populations and just 1 or 2 peaks for monoclonal ones.

Heteroduplex analysis uses double-stranded PCR products which are separated according to their length and conformation, whereas GeneScan analyzes single-stranded products which are separated only according to their length. In the case of a sample with a monoclonal lymphoid population, a variable proportion of homoduplexes will be formed. Visualization by polyacrylamide gel electrophoresis reveals a band corresponding to an appropriate length. Samples containing a high proportion of polyclonal cells produce abundant heteroduplexes with little likelihood of homoduplex binding.

GeneScan can distinguish between amplified products that differ by a single bp, thereby allowing the detection of the same clone in different samples or tissues without the need for sequencing. The heteroduplex analysis is considered to have greater sensitivity and specificity than the GeneScan system, particularly when amplifying Ig κ , κ de, and Ig λ rearrangements. In the analysis of DJ segments of the IgH gene, heteroduplex analysis or GeneScan can be used interchangeably.¹⁵

Fluorescent Fragment Analysis (GeneScan)

In the GeneScan system, 1 of the primers is labeled with fluorochrome. After amplification, the PCR products are discriminated or separated in a polymer capillary electrophoresis system in a process that involves mixing 1

μ L of PCR product with 0.5 μ L of a standard molecular weight product (GeneScan 400-ROX, Applied Biosystems, Foster City, CA, USA) in 9 μ L of deionized formamide for denaturing into single DNA strands. The products are detected automatically by fluorescence reading with a laser system in an automatic DNA sequencer (ABIPrism 3100, Applied Biosystems). A result is considered monoclonal if 1 or 2 peaks of amplified product with an appropriate bp length for the PCR in question are obtained. Detection of 3 to 5 peaks is considered an oligoclonal result, and more than 5 peaks of different lengths would be interpreted as a polyclonal result. To consider a result as a truly monoclonal, the height of the peak should be at least twice that of the normal distribution of the polyclonal peaks, which correspond to the presence of reactive cells in the infiltrate (Figure 4).

The overall sensitivity for detection of B- or T-cell clonality of the BIOMED-2 protocols which use GeneScan for reading is approximately 0.5%-5%. The limits of detection depend on the technique used, the quality of the sample (paraffin-embedded samples have a lower yield), and the proportion of reactive lymphoid cells present in the sample. The technique is considered sufficiently sensitive with detection of a clonal population that comprises 5%-10% of the total lymphoid population of the infiltrate.

Heteroduplex Analysis

The PCR products are denatured by heating (95°C for 5 minutes) followed by rapid renaturation at low temperature (4°C for 1 hour) in an attempt to facilitate renewed spontaneous and, in this case, random hybridization in such a way that homoduplex or heteroduplex DNA fragments are obtained with a different conformation. In the case of heteroduplexes, the different conformations obtained give rise to different physical

Table 1. BIOMED-2 Polymerase Chain Reaction (PCR) Protocols

<p>For V_H PCR according to the BIOMED-2 protocol, 3 sets of primers are used—each containing 6 or 7 primers—1 each for framework regions (FR) I, FRII, and FRIII—able to hybridize to the corresponding V_H segments (V_H1-7). A simple JH set able to hybridize to the 6 functional JH segments is used. The expected V_H PCR product contains 100-170 bp, 250-295 bp, and 310-360 bp for FRIII, FRII, and FRI, respectively. D_H PCR uses 7 primers, 1 per family, and the same JH set for V_H PCR. The expected PCR product has between 100 and 420 bp. The VJ Igk gene is 120-300 bp long and the VJ Igg gene 140-160 bp long. In addition, the Igk locus can undergo rearrangement of the kde segment directly to Vk, with a length of 210-390 bp. This rearrangement deactivates the Igk allele. The size of the TCRg PCR products is 231-283 bp for group I, 147-207 bp for group II, 132-182 bp for group III, and 116-165 bp for group IV. The size of the final β rearrangement product is approximately 170-325 bp, depending on the set of primers used.</p>
PCR Reagents
100 ng DNA
10 pmol of each primer (fluorochrome labeled or not depending on the reading system used). The number and type of primer for each tube depends on each specific PCR.
Nucleotides for a final concentration of 200 μM
MgCl ₂ at a final concentration of 1.5 μM
1-2 units of Taq polymerase (AmpliTaq Gold [Applied Biosystems, Foster City, CA, USA]), depending on whether or not the PCR tube has more or less primers
ABI Buffer II or ABI Gold (AmpliTaq Gold [Applied Biosystems]) for a final volume of 50 μL
PCR Conditions
Preactivation of Taq polymerase for 7 min at 95°C
Hybridization temperature: 60°C
Number of cycles: 35
Cycle phases: Denaturing: 30 (to 45) s (new PCR systems are faster) Hybridization: 30 (to 45) s Extension: 30 s (to 1.5 min)
Final 10 min extension
Product storage possible at room temperature or at 15°C

characteristics, and so they can be separated by means of non-denaturing polyacrylamide gel electrophoresis. Homoduplexes achieve a perfect hybridization and migrate more quickly. The PCR products corresponding to monoclonal samples form a high proportion of homoduplexes that are mixed with heteroduplexes obtained from other polyclonal or reactive lymphocytes present in the sample and these are clearly separated into bands in the polyacrylamide gel. The polyclonal samples only give rise to heteroduplexes (Figure 5).

Heteroduplex analysis is rapid and simple, and has an overall sensitivity for the detection of B- or T-cell clonality of approximately 1%-10% (using the BIOMED-2 protocols), even when archival paraffin-embedded samples are studied.

Sensitivity and Specificity

False Negatives (Sensitivity)

Failure to detect clonality may occur when the molecular weight of the fragment to be amplified is greater than that indicated by the DNA quality control PCR. The use of multiple primers designed for the BIOMED-2 project for different segments can minimize the number of false negatives that arise from hybridization errors due to lack of specificity or homology with the primers, particularly when studying B-cell clonality, in which somatic hypermutations in the V_H segment can cause hybridization failure.¹⁹

False Positives (Specificity)

Detection of false positives, or pseudoclonality, becomes particularly important in cases in which the lymphoid infiltrate has reactive characteristics, with scant cellularity, or when small samples are studied.³² This is a crucial element in the BIOMED-2 protocols, and to minimize this phenomenon, it is recommended to carry out the analysis in duplicate and, if possible, to study more than one sample from the same patient. Some authors recommend that this particular type of sample should be analyzed simultaneously using GeneScan and heteroduplex analysis, given the higher rate of false positives for GeneScan in such situations.

If the same clonality peak cannot be reproduced, the results should be considered as negative or pseudoclonal. It is also recommended to check in duplicate the clonality of a lymphoproliferative process by detecting a dominant clone using different Ig and TCR targets or gene segments.

Finally, it should be remembered that detection of a monoclonal rearrangement of the TCR or Ig gene cannot unequivocally be interpreted as marker of a specific cell line or population. If the exceptional case of a composite lymphoma is ruled out, double aberrant T- and B-cell

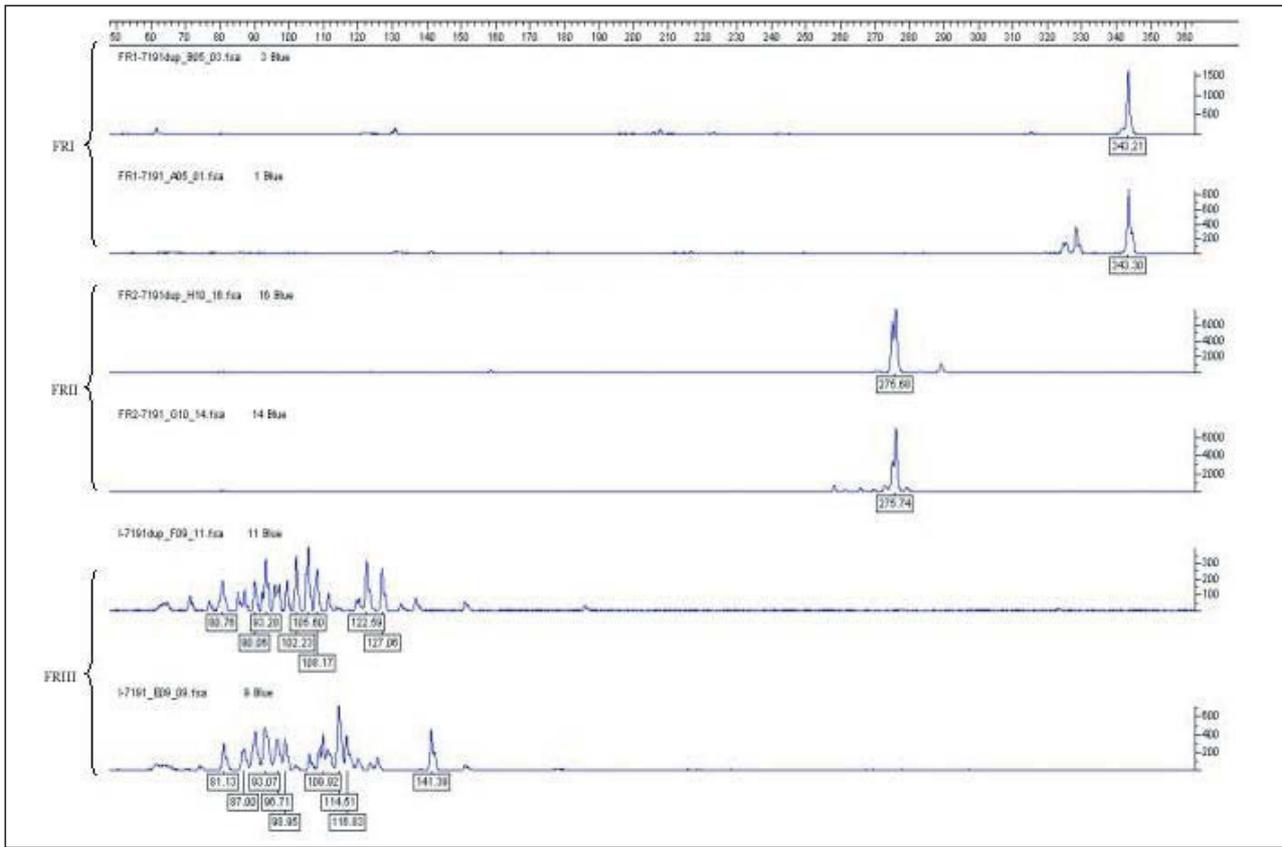


Figure 4. GeneScan automated reading system for polymerase chain reaction fragments. The figure shows a monoclonal result by framework region (FR) II and FRI analysis and a polyclonal result by FRIII analysis.

rearrangements or cross rearrangements (cross lineage) may be detected in the same sample, particularly in immature cell malignancies, although this can also be seen in 5%-10% of mature cell neoplasms.³³

Study of T-Lymphoid Clonality

Technical and Methodological Aspects

Amplification of the VJ gene of the TCR γ subunit by PCR is the most widely used method to evaluate clonality of an infiltrate when a T-cell origin is suspected.^{4,8,10-12,23,26,29,30,34,35} The technique is relatively simple, given that the gene rearrangement involves a limited repertoire of gene segments. The length of the amplified segment (approximately 200 bp) can yield results in most archival tissue samples. The TCR γ gene is rearranged in most T cells irrespective of whether they express TCR $\alpha\beta$ or TCR $\gamma\delta$ on their surface.^{15,36,37}

PCR analysis of the complete VDJ gene or incomplete DJ gene that encodes the TCR β subunit is a more complex technique.^{9,38,39} The TCR β subunit has a greater number

of combinatorial possibilities, so more primers are needed, and the amplifiable segments are much longer or heavier (Figure 6). The TCR β gene is rearranged not only in all TCR $\alpha\beta$ cells but also in many TCR $\gamma\delta$ ones.^{15,36,37} PCR analysis of the TCR β gene is a complementary test that should be done when there is reasonable suspicion of T-cell lymphoma and PCR-TCR γ results are inconclusive. By combining such studies, T-cell clonality can be detected in almost all samples of T-cell lymphoma.¹⁵

Likewise, TCR δ gene rearrangements can be detected. This is useful both in the study of immature lymphoid malignancies and in lymphoproliferative processes arising from TCR $\gamma\delta$ cells.^{36,37} As a result of its complexity (due to the a high number of combinatorial possibilities with segments widely spaced on the genome), the TCR α gene is not included in the BIOMED-2 project.¹⁵

Results for Cutaneous T-Cell Lymphoma

Most studies that use electrophoresis gels detect clonality in between 40% and 90% of the samples of cutaneous T-cell lymphoma (CTCL).³⁵ Different technical aspects, type

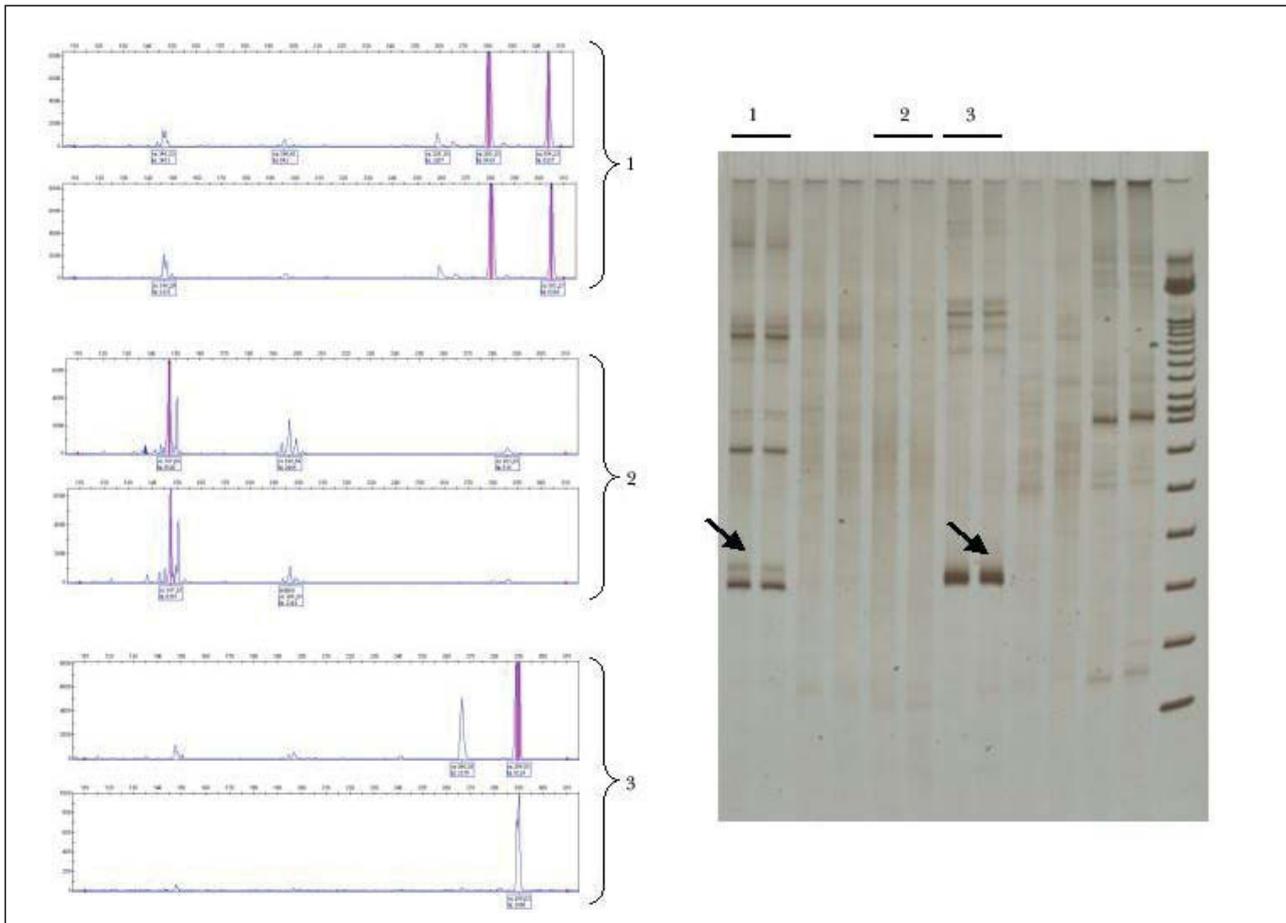


Figure 5. Heteroduplex analysis. Heteroduplex analysis can be more informative than GeneScan for certain rearrangements such as in the case of Ig λ in the figure.

of reading system of the amplified products, and sample characteristics (paraffin-embedded or frozen, differing proportions of early and advanced stages) might explain the differences between the different series. Monoclonal lymphoid proliferation is usually detected more often in advanced stages of mycosis fungoides and in Sézary syndrome (90%-100%) compared to early mycosis fungoides lesions (50%-70%).^{8,10-12,23,26,29,30,34,35,38-42}

With application of PCR techniques and highly sensitive and specific reading systems (GeneScan, heteroduplex analysis), lymphoid monoclonality can be detected in approximately 60%-70% of CTCL samples.^{8,12,23,26,29,30,40-42} In the series reported by Ponti and coworkers,³⁵ 162 of the 194 samples of CTCL (83.4%) were found to be clonal using PCR amplification of TCR γ and heteroduplex analysis.³⁵ Similar results were obtained by the same authors using the GeneScan system (84%).³⁴ There appears to be a good correlation between the characteristics of the infiltrate (density, epidermotropism), immunophenotype (loss of T-cell antigens), and stage when the presence of a dominant

clone can be detected. Unlike most studies, Cherny et al⁴¹ only detected 42% of clonal samples with heteroduplex analysis in a series of CTCL; however, this result does not appear to be representative because of the small number of cases analyzed (n=7).

Application of the BIOMED-2 protocols with the GeneScan system has achieved high sensitivity in the study of different cutaneous T-cell lymphoproliferative processes. Although results are lacking in large series with paraffin-embedded samples, T-cell clones have been detected in between 63% and 85% of cutaneous samples of mycosis fungoides/Sézary syndrome by PCR with GeneScan of TCR γ and in between 63% and 75% by PCR with GeneScan of TCR β .^{38,42}

A dominant clone is detected slightly less often in CD30⁺ lymphoproliferative processes than in other CTCL, perhaps because of the specific characteristics of a lymphomatous infiltrate that usually has a heterogenous cell population.⁴³⁻⁴⁵ In our experience, half of the samples of lymphomatoid papulosis from frozen samples are clonal, as well as two-

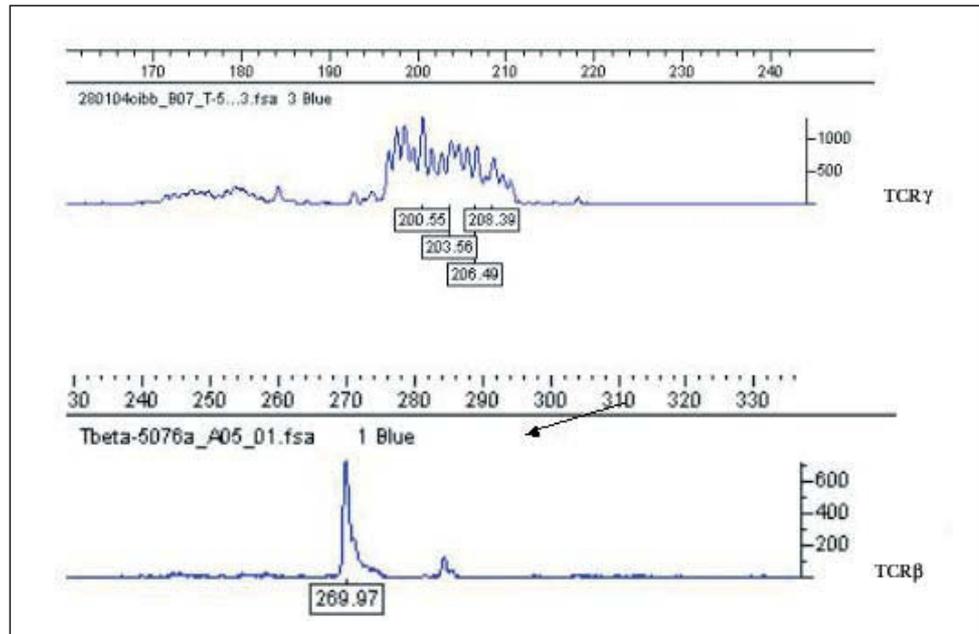


Figure 6. Rearrangement of the TCR γ gene. The figure shows a clonal TCR β result not detected by TCR γ .

thirds of CTCL CD30⁺ samples. In a recent study of paraffin-embedded samples of lymphomatoid papulosis and CD30⁺ CTCL by PCR with GeneScan, a T-cell clone was detected in only 22% of the cases of lymphomatoid papulosis (4/18), particularly in those forms with a high proportion of atypical cells.⁴⁶ In that study, the detection rate was 65% for CD30⁺ CTCL samples.

Those results indicate that an important feature of CTCL diagnosis is demonstrating a dominant clone given that the presence of a polyclonal rearrangement pattern is uncommon. However, the presence of a T-lymphocyte clone has also been detected in a variable number of samples from chronic inflammatory dermatoses that were considered benign but that showed an uncertain relationship with subsequent development of actual CTCL.^{39,47,48} Some authors have put forward the concept of “cutaneous lymphoid dyscrasias” to denote this group of processes.⁴⁹ Lichenoid pityriasis, certain cases of pigmented purpuric dermatoses, atypical lobular panniculitis, certain drug reactions, and even a group of entities considered as “parapsoriasis” would be included in such a group.

The differential diagnosis for parapsoriatic lesions, defined as persistent erythematous-desquamative plaques without histopathologic criteria for mycosis fungoides, with respect to early mycosis fungoides lesions can pose substantial practical difficulties. There is certain controversy concerning the rate of detection of T-cell clonality in such cases. Some authors detect T-lymphocyte clonality in almost 50% of the samples, and suggest that these cases be considered as premycosis fungoides lesions or truly incipient mycosis fungoides lesions. However, other authors almost never

detect clonality.⁵⁰⁻⁵⁴ The reason for these discrepancies is probably the lack of standard criteria for defining the concept of parapsoriasis (Table 2).⁵⁵ In our experience, application of PCR for TCR γ can detect clonality in parapsoriatic lesions in a very few cases—less than 5%—whereas approximately 70% of the cases of mycosis fungoides in early stages are found to be clonal.⁵⁶

Finally, GeneScan reading can be used to assess clonality agreement between samples of different lymphoid processes that occur simultaneously or successively in the same patient (for example, mycosis fungoides and lymphomatoid papulosis lesions). If it is the same clone that is being detected, the monoclonal peak should be identical and correspond to the same length; this finding obviates the need to sequence the PCR products.^{57,58}

Data published on detection rates of a dominant clone in reactive T-lymphocyte proliferative processes is very variable (0%–24%),⁵⁹⁻⁶¹ and comes mainly from studies that use traditional electrophoresis systems. Ponti and coworkers³⁵ were able to detect a dominant clone in up to 2% of the samples in an extensive series of reactive T-cell samples, reflecting the good specificity of heteroduplex analysis (97.7%); the technique is particularly useful for distinguishing between erythrodermas of inflammatory origin and Sézary syndrome. The specificity of the BIOMED-2 protocols in the analysis of cutaneous samples of reactive origin or characteristics is high.⁴² Thus, faced with the need to confirm the presence of a T-cell clone in a reactive lymphoid infiltrate, study of the TCR β gene, in view of its larger gene repertoire, may offer greater specificity.³⁹

Table 2. Criteria for Diagnosis of Incipient Mycosis Fungoides (International Society of Cutaneous Lymphoma)

Criteria	Major (2 Points)	Minor (1 Point)
Clinical	2 y	1 y
Persistent and/or progressive Macules or plaques + Unexposed area Various shapes/sizes Poikiloderma		
Histologic	Both	Any
Superficial lymphoid infiltrate + Epidermotropism without spongiosis Atypia		
Molecular		Present
Monoclonal T-cell receptor rearrangement		
Immunohistochemical		1 y
CD2, CD3, CD5 <50% CD7 <10% Discontinuity between epidermis and dermis		

Definitive diagnosis of mycosis fungoides is made for a score of 4 points or more.

Study of B-Lymphoid Clonality

Technical and Methodological Aspects

Study of rearrangement of the IgH gene is done using V_H and J_H consensus primers for the framework regions (FR) I, FRII, or FRIII. The FRIII-J_H segment is the one most often studied for technical reasons; namely, the number of combinatorial possibilities is lower and the 2 segments are very close together in the rearranged gene (approximately 100 bp).

In an appropriate clinical-pathological context, in cases in which B-lymphoid clonality is not detected after PCR amplification of the FRIII-J_H segment, study of the FRII and FRI segments is recommended. However, the FRI segment is long and many more primers are needed for the study, making it often unfeasible to work with paraffin-embedded samples.¹⁵

The BIOMED-2 protocols include not just analysis of the complete VDJ gene of the IgH gene, but also the design of a PCR for studying incomplete DJ rearrangement.¹⁵ This segment is less subject to somatic hypermutations, which are common processes in mature B-cell malignancies.¹⁹ The high number of somatic hypermutations—which occur in

V_H segments during transit of B cells through the germinal center—is responsible for a greater likelihood of primer hybridization errors and, therefore, of false negative results.

PCR methods have been described for amplification of the Igλ and Igκ genes, which in certain circumstances can yield greater sensitivity in the detection of B-cell clonality.⁶² Furthermore, κde segment rearrangement, which is also unaffected by somatic hypermutations, can be analyzed in addition to study of the complete Igκ gene. Rearrangement of κde is usually present in all Igλ malignancies and one-third of those of Igκ origin. Study of incomplete DJ rearrangement, as well as of rearrangement of the Igλ, Igκ, and κde genes, is used in those cases in which it has proved impossible to demonstrate the presence of a monoclonal B-cell population after analysis of the complete IgH (VDJ) gene (Figure 7).¹⁵

Results in Cutaneous B-Cell Lymphomas

Study of B-lymphoid clonality by PCR of the FRIII region can detect clonality in 30%-70% of B-cell lymphomas, depending on the type of sample and the laboratory technique used.^{1,27,63,64} Application of the BIOMED-2 protocol in sufficiently high-quality samples of systemic B-cell lymphomas, with simultaneous study of the FRI, FRII, and FRIII regions, managed to detect a clonal pattern in up to 90%-95% of cases. The presence of somatic hypermutations probably explains why clonality is not detected in all cases.^{15,16}

The combination of study of complete IgH rearrangement (VDJ) with study of incomplete DJ rearrangements, as well as rearrangement of the light chains, has achieved almost 100% detection of clonality in cases of mantle-cell lymphomas, chronic lymphocytic leukemias, follicular lymphomas, and marginal zone lymphomas, and up to 98% detection in diffuse large B-cell lymphomas.^{15,16}

For cutaneous B-cell lymphomas (CBCL), different studies have detected a sensitivity between 34% and 83% using PCR for B-cell clonality, generally with electrophoresis reading systems.^{42,63,65-70} It should be remembered that most of the studies published only analyze the FRIII region or segment, and so the rate of false negatives may be as high as 50%.⁶⁴

Gellrich et al,⁷¹ in a recent study of paraffin-embedded samples of CBCL using BIOMED-2 protocols for amplification of the complete VDJ IgH gene (FRIII, FRII, FRI), found an overall clonality rate of 55.8% (100% for CBCL of the leg, 54.5% for marginal zone CBCL, and 66.7% for follicular CBCL).⁷¹ In that study, PCR for FRIII detected 46.5% clonality (100% for CBCL of the leg, 36.4% for marginal zone CBCL, and 40.7% for cutaneous follicular lymphomas). PCR of FRI only detected 20.9%, taking into account all samples (for 76% of the paraffin-embedded

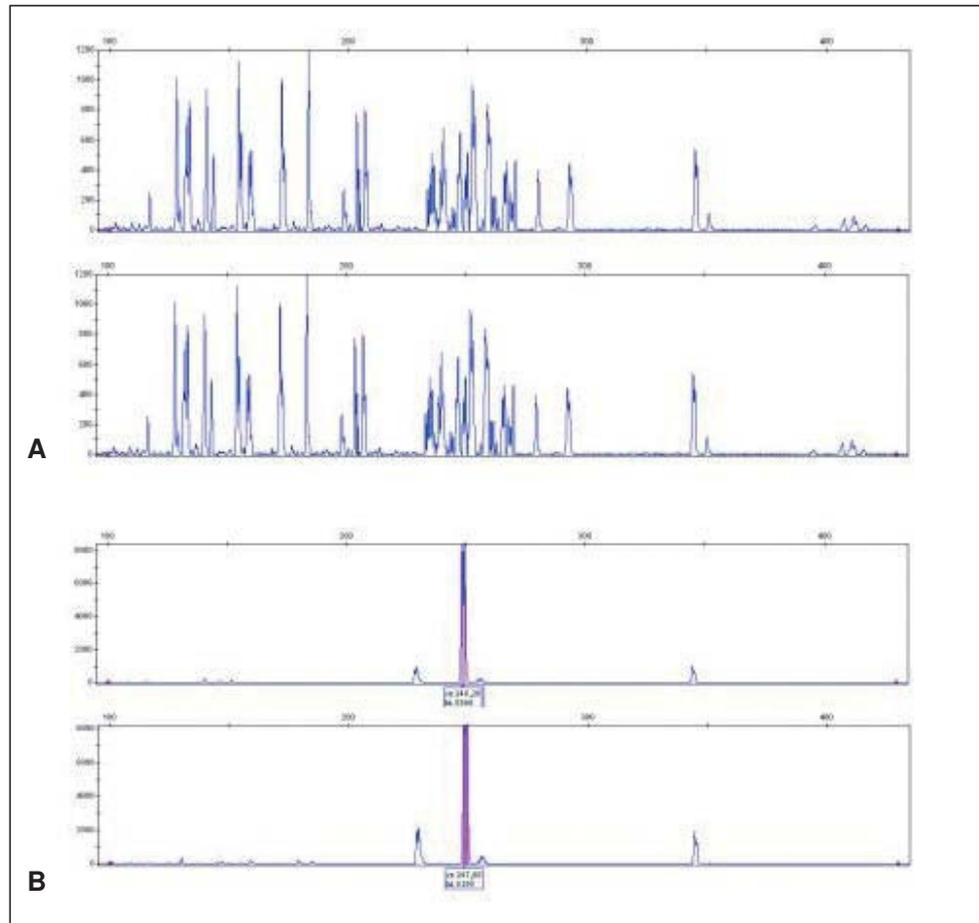


Figure 7. Study of incomplete DJ rearrangement of the IgH gene. DJ clonal result (B) not detected in the study of the complete VDJ gene (A).

samples, the level of quality control was below that recommended for amplification of the FRI fragment). However, if only amplifiable samples only were considered, PCR of FRI achieved a higher clonality detection rate (90%), which indicates that it is an excellent technique, particularly in fresh or frozen samples. Study of other FR regions seems to significantly increase sensitivity and would probably do so to a larger extent if incomplete DJ, Ig λ , Ig κ , and κ de rearrangements were also added to the B-cell genotypic analysis.¹⁵

Conclusion

The BIOMED-2 project for clonality analysis by GeneScan and heteroduplex analysis has proved to be a diagnostic strategy of high sensitivity and specificity for studying patients with cutaneous lymphoproliferative processes. Quality control and amplifiable DNA, particularly when obtained from material embedded in paraffin, is crucial for establishing whether a particular sample can be analyzed by a given PCR technique. The PCR study should be done in duplicate for each sample and, if possible, for 2 different

samples from the same patient. If the clonality peak of the sample cannot be reproduced (with the same length), the result is not considered positive. This helps avoid the erroneous interpretation that a sample is monoclonal (false positives or pseudo-clonality).

In the analysis of clonality of a lymphoid infiltrate of T cells, PCR amplification of TCR γ is studied initially. This gene, being a prototypical example of a short segment with few variable regions, is well preserved in archival paraffin-embedded samples. TCR γ is rearranged early in most T-cell malignancies, regardless of whether the phenotype is TCR $\alpha\beta$ or TCR $\gamma\delta$. Given the greater complexity and laboriousness of the technique, study of rearrangement of the TCR β gene represents a complementary strategy for those cases in which detection by TCR γ amplification has failed.

A strategic approach to sequencing in the study of B-cell clonality would be to start with study of the IgH gene in its FRIII segment first and subsequently study the FRII and FRI segments. Although the rates of detection of incomplete DJ, Ig κ , Ig λ , and κ de rearrangements are lower than in study of the complete VDJ gene, these are interesting complementary targets because they are unaffected by

hypermutations, which may be encountered particularly in marginal zone CBCL and follicular center CBCL.

Conflicts of Interest

The authors declare no conflicts of interest.

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