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ORIGINAL ARTICLE

Improved reliability of lymphoma diagnostics via PCR-based clonality testing: — Report of the BIOMED-2 Concerted Action BHM4-CT98-3936

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The diagnosis of malignant lymphoma is a recognized difficult area in histopathology. Therefore, detection of clonality in a suspected lymphoproliferation is a valuable diagnostic criterion. We have developed primer sets for the detection of rearrangements in the B- and T-cell receptor genes as reliable tools for clonality assessment in lymphoproliferations suspected for lymphoma. In this issue of *Leukemia*, the participants of the BIOMED-2 Concerted Action CT98-3936 report on the validation of the newly developed clonality assays in various disease entities. Clonality was detected in 99% of all B-cell malignancies and in 94% of all T-cell malignancies, whereas the great majority of reactive lesions showed polyclonality. The combined BIOMED-2 results are summarized in a guideline, which can now be implemented in routine lymphoma diagnostics. The use of this standardized approach in patients with a suspect lymphoproliferation will result in improved diagnosis of malignant lymphoma.
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Introduction: relevance of clonality testing

The knowledge explosion in biomedical research, especially in the field of cancer, now leads to the improved treatment and survival for the cancer patients. This progress in clinical oncology asks for early diagnosis of cancer with high accuracy. A paradoxical situation exists for malignant lymphomas. More effective treatment options lead to a better long-term survival and increased cure rates in lymphoma patients, but the diagnosis and classification of malignant lymphomas is a well-recognized problematic area for pathologists.[1,2]

A key feature of cancer is the monoclonality of the tumor cells, as all tumor cells are the progeny of a single malignantly transformed cell. This characteristic enables the discrimination between polyclonal, reactive processes and monoclonal, malignant tumors.[3–6] Clonality testing can, in principle, be used in all lymphoproliferations, as the key feature of lymphocytes is that they have rearranged antigen-receptor genes, which are unique for each lymphocyte.[3–6] The stepwise rearrangement process in each immunoglobulin (Ig) or T-cell receptor (TCR) gene during early lymphoid differentiation joins V-, D- and J-gene segments out of the many available segments. During the rearrangement process, nucleotides are deleted and randomly inserted at the joining sites, resulting in an enormous diversity of antigen receptors (Figure 1).[7,8] Reactive lymphoproliferations therefore have polyclonally rearranged Ig or TCR genes, whereas malignant lymphoproliferations show clonal rearrangements.[3,9] This knowledge has led to the use of Ig/TCR clonality assessment as a tool for diagnosing malignant lymphoma with high certainty, although it is essential to realize that monoclonality is not always equal malignancy.[3,9–13]

In B-cell lymphomas, Ig light chain restriction (Igκ or Igλ expression) has been used as a surrogate marker for clonality for many years.[14,15] This still is a reliable method in lymphomas with plasma cell differentiation, because these lymphomas have sufficient amounts of Ig molecules to be detected in formalin-fixed paraffin-embedded tissue. Its use in other types of B-cell lymphomas requires a very sensitive technique, and is not reproducibly performed in many laboratories.[16] However, Southern blot analysis of Ig- and TCR-gene rearrangements has been considered the gold standard method for clonality testing.[10,17–19] Despite its high reliability, the main drawback of Southern blot analysis is the necessity for large amounts of

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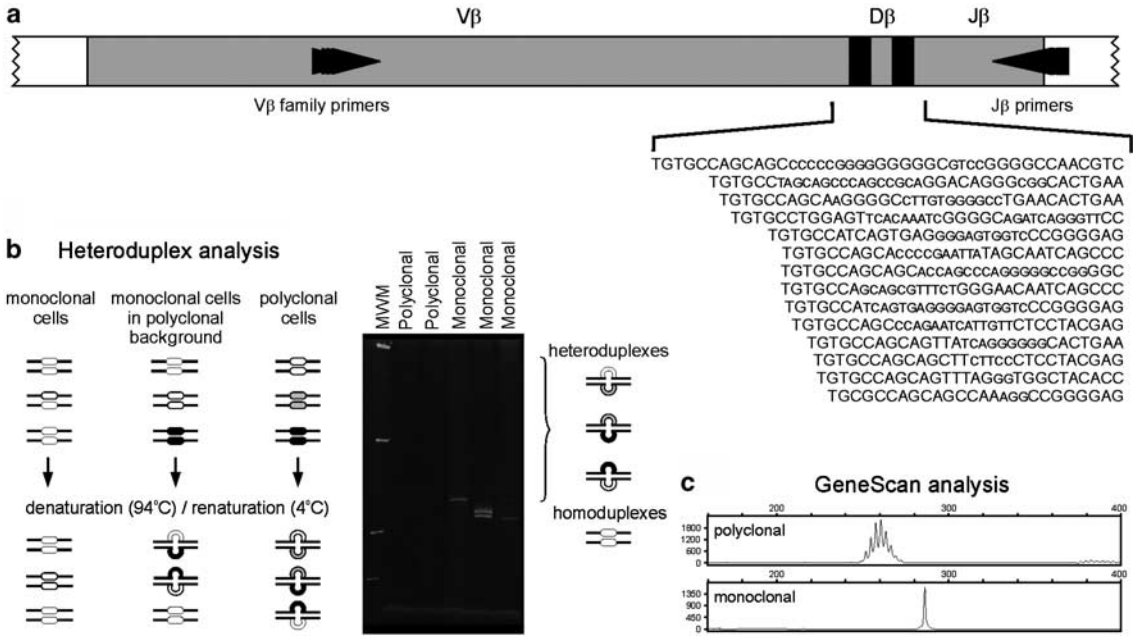

Figure 1 Schematic diagram of heteroduplex analysis and GeneScan analysis of PCR products from Ig/TCR-gene rearrangements. (**a**) Rearranged Ig/TCR genes (here *TCRB* rearrangements are shown as example) show heterogeneous junctional regions that differ in size and nucleotide composition. V, D and J germ-line nucleotides are shown in large capital, and randomly inserted nucleotides in small capital. Junctional heterogeneity can be exploited to discriminate between polyclonal and monoclonal PCR products using heteroduplex analysis or GeneScan analysis. (**b**) In heteroduplex analysis, PCR products are denatured (5′, 94°C) and reannealed (rapid cooling at 4°C for 60 min).[18,27] Monoclonal PCR products give rise to homoduplexes, whereas polyclonal PCR products mainly form heteroduplexes, resulting in a smear of slow-migrating fragments. (**c**) In GeneScan analysis, fluorochrome-labeled PCR products are denatured for high-resolution fragment analysis of the single-stranded fragments.[27,34] Monoclonal PCR products of identical size give rise to a peak, whereas polyclonal PCR products show a Gaussian size distribution.

high molecular weight DNA extracted from fresh or frozen tissue, that is often not available in daily diagnostic practice. Furthermore, it is a technically demanding and a time-consuming method.

For this reason, polymerase chain reaction (PCR)-based methods for clonality testing have been developed, as they are fast and require only limited amounts of medium quality DNA.[20–25] Most of the published methods use consensus primers for completely rearranged antigen receptor genes, mainly the Ig heavy chain (*IGH*) and TCR gamma (*TCRG*) genes. However, worldwide many different PCR protocols and many different primers are in use, each with different sensitivity and applicability. Consequently, these protocols and primers can give contradictory results, particularly in suspected T-cell proliferations.[24–26] False-negative results in PCR-based clonality studies are mainly due to lack of sufficient primers that cover the many V-, D- and J-gene segments or due to improper annealing as a consequence of somatic hypermutation in Ig genes. False-positive results are particularly caused by the difficulties in discrimination between PCR products derived from monoclonal versus polyclonal Ig- and TCR-gene rearrangements.[27]

Therefore, the BIOMED-2 Concerted Action BMH4 CT98-3936 was initiated to develop standardized reagents and methods for PCR-based clonality diagnostics.[27] A total of 47 institutes from seven European countries collaborated in this project, exploiting the full knowledge of Ig and TCR genes and their rearrangement processes. This resulted in highly efficient multiplex PCR protocols using multiple primers for virtually all different functional gene segments of the Ig and TCR genes.[27–29] After initial testing of the multiplex PCR tubes,[27] a large series of almost 400 B-cell malignancies[30] almost 200 T-cell malignancies[31] as well as more than 100 histomorphologically reactive lesions[32] were evaluated to cover the spectrum of diagnostic situations in hematopathology. All lymphoma cases were diagnosed according to the WHO criteria;[33] the international BIOMED-2 Pathology Review Panel supervised this process of diagnosis and classification (see supplementary website figures in Evans *et al*.[30] and Brüggemann *et al*.[31]).

The BIOMED-2 results demonstrate that the use of these standardized PCR protocols and primer sets is feasible and reliable in routine practice and leads to the improved and potentially early diagnosis of malignant lymphomas. In this era of increased attention for quality improvement in medicine, we believe that the introduction of the BIOMED-2 clonality assays lead to a more reliable diagnosis and thus to a better care for patients who are suspected to have a malignant lymphoma. In this report, we summarize the data from the three large-scale BIOMED-2 studies[30–32] and present the consequent guideline for efficient clonality diagnostics.

BIOMED-2 multiplex tubes and protocols

In order to avoid false-negative PCR results, it was decided to include *IGH*, *IGK* and *IGL* genes as well as *TCRB*, *TCRG* and *TCRD* genes as complementary PCR targets. *TCRA* was not included because of its high level of complexity. A total of 97 new primers were designed, covering the majority of functional gene segments and representing 418 single PCR tests. After initial evaluation of all separate 418 PCR tests, careful combinations of the primers resulted in only 14 Ig/TCR multiplex PCR tubes: three for complete *IGH* (VH–JH), two for incomplete *IGH* (DH-JH), two for *IGK* (Vκ-Jκ and Kde rearrangements), one for *IGL* (Vλ-Jλ), two for complete *TCRB* (Vβ-Jβ), one for incomplete *TCRB* (Dβ-Jβ), two for *TCRG* (Vγ-Jγ) and one for all types of *TCRD* gene rearrangements (available

from *in vivo* Scribe Technologies, San Diego, CA, USA; www.invivoscribe.com).[27] The multiplex PCR analyses were performed according to the fully standardized PCR protocols.[27]

Following amplification, the obtained Ig/TCR PCR products were subjected to heteroduplex analysis or GeneScan fragment analysis as preferred methods for discrimination between monoclonal and polyclonal PCR products.[20,27,34] In case of reactive non-clonal proliferations, heteroduplex and GeneScan analysis result in polyclonal smears and Gaussian curves, respectively, whereas clonal lymphoid cell populations result in clear bands or peaks of monoclonal PCR products, respectively (Figure 1).

High clonality detection rates

The BIOMED-2 multiplex PCR tubes detected clonal Ig- or TCR-gene rearrangements in the vast majority of lymphoid malignancies (99% of all B-cell malignancies and 94% of all T-cell malignancies; Tables 1 and 2),[30,31] whereas the reactive lesions were most often polyclonal in nature (>90%).[32] The highly diverse *TCRB* gene was included as PCR target for clonality testing with clonal results in >90% of T-cell malignancies.[31] The strength of the BIOMED-2 *TCRB* assay is its coverage of 325 single PCR tests using 23 Vβ-, 13 Jβ- and two Dβ-primers in only three well-attuned multiplex tubes.[27,29] Therefore, the informativity of *TCRB* in T-cell malignancies was comparable to the informativity of *IGH* in B-cell malignancies with a clonality detection rate of approximately 90%.[30,31] Other important results included the detection of 100% Ig-gene rearrangements (*IGH* and *IGK*) in the heavily somatically mutated follicular lymphomas and marginal zone lymphomas and the detection of occult lymphomas in lesions considered reactive by expert pathologists.[30,32]

The strength of our PCR approach appeared to be the use of complementarity at the three levels. Firstly, the many new primers were designed to recognize the majority of functional Ig and TCR gene segments and to fit in a limited number of multiplex PCR tubes. Secondly, for the *IGH* and *TCRB* loci not only complete V–D–J rearrangements were included as PCR targets, but for the first time we also used incomplete DH–JH and Dβ–Jβ rearrangements, which were detected in ~30% of B-cell malignancies and in ~60% of T-cell malignancies, respectively (Tables 1 and 2).[30,31] Thirdly, the usage of at least two Ig or TCR loci in parallel appeared to be highly effective, that is, *IGH* and *IGK* for B-cell malignancies (Table 1)[30] and *TCRB* and *TCRG* for T-cell malignancies (Table 2).[31] The reports by Evans *et al*. and Brüggemann *et al*.,[30,31] in this issue of *Leukemia*, present the complete data set concerning the detection of Ig- and TCR-gene rearrangements in B-cell malignancies and T-cell malignancies.

Clonal *IGK*-gene rearrangements were found in ~90% of all B-cell malignancies, which is in line with the high frequency of *IGK* (Vκ–Jκ) and *IGK*-Kde rearrangements in normal Igκ^+ and Igλ^+ B cells.[36,37] Consequently, the combined *IGH* and *IGK* clonality detection rate reached the unprecedentedly high frequency of 99% (Table 1).[30] Analogously, *TCRG*-gene rearrangements were found in ~90% of T-cell malignancies, which is in line with the presence of *TCRG*-gene rearrangements in the vast majority of normal T cells of the TCR$\alpha\beta$ lineage from which most T-cell malignancies are derived.[27,38] The observation that in 20–25% of anaplastic large-cell lymphomas (ALCL), no *TCRB* and *TCRG* rearrangements detected (Table 2) that fits with the Southern blot results in this patient group.[35] If we thus consider ALCL to be an extraordinary type of T-cell malignancy, the combined *TCRB* and *TCRG* targets in the more typical T-cell malignancies reach the unprecedentedly high clonality detection rate of 99%.[31]

The application of multiple complementary targets in parallel does not only reach high overall clonality detection rates, but also has the advantage that in more than 90% of the cases at least two clonal results are obtained and in 70–80% even three or more.[30,31] The BIOMED-2 multiplex tubes can detect the same rearrangement more than once (e.g. *IGH*) and can detect biallelic rearrangements, two different rearrangements on the same allele (e.g. Vκ–Jκ and Kde or Vβ–Jβ and Dβ–Jβ), and rearrangements in more than one Ig or TCR gene of the same clone.[39,40] In practice, the multiple clonal results can be achieved by the use of only five Ig tubes and five TCR tubes (Figure 2). This makes the BIOMED-2 multiplex tubes into a highly reliable and feasible system for clonality diagnostics.

TCR-gene rearrangements occur in 10–20% of B-cell malignancies (generally found in a single TCR locus) and Ig-gene rearrangements occur in 5–10% of T-cell malignancies.[30,31] Thus, formally individual Ig- and TCR-gene rearrangements cannot be used as markers for B/T-lineage assignment, but the complete Ig/TCR-gene rearrangement pattern of a lymphoid malignancy might support lineage assignment. It should be

Table 1 Complementarity of Ig targets for clonality detection in five categories of B-cell malignancies (% clonality)[a]

	IGH			*IGK*	*IGH+IGK*
	VH–JH	*DH–JH*	*VH–JH+DH–JH*	*Vκ–Jκ+Kde*	
MCL(%)	100	11	100	100	100
B-CLL(%)	100	43	100	100	100
FL(%)	84	19	86	84	100
MZL(%)	88	51	95	83	100
DLBCL(%)	79	30	85	80	98
TOTAL(%)	88	28	91	88	99

Abbreviations: B-CLL, B-cell chronic lymphocytic leukemia; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; MCL, mantle cell lymphoma; MZL, marginal zone B-cell lymphoma.
[a]For detailed information, see BIOMED-2 report by Evans *et al*.[30]

Table 2 Complementarity of TCR targets for clonality detection in five categories of T-cell malignancies (% clonality)[a]

	TCRB			*TCRG*	*TCRB+TCRG*
	Vβ–Jβ	*Dβ–Jβ*	*Vβ–Jβ+Dβ–Jβ*	*Vγ–Jγ*	
T-PLL(%)	94	47	100	94	100
T-LGL(%)	86	62	96	96	100
PTCL-U(%)	85	67%	98	94	100
AILT(%)	70%	61	89	92	95
ALCL(%)	70	48	74	74	79[b]
Total(%)	80	58	91	89	94 (99)[b]

Abbreviations: ALCL, anaplastic large-cell lymphoma; AILT, angioimmunoblastic T-cell lymphoma; PTCL-U, peripheral T-cell lymphoma, unspecified; T-LGL, T-cell large granular lymphocytic leukemia; T-PLL, T-cell prolymphocytic leukemia.
[a]For detailed information, see BIOMED-2 report by Brüggemann *et al*.[31]
[b]Approximately 20–25% of ALCL are known to have no TCR gene rearrangements and are defined as null ALCL.[35] If the ALCL are excluded from the calculations, the clonality detection rate in T-cell malignancies is 99%.

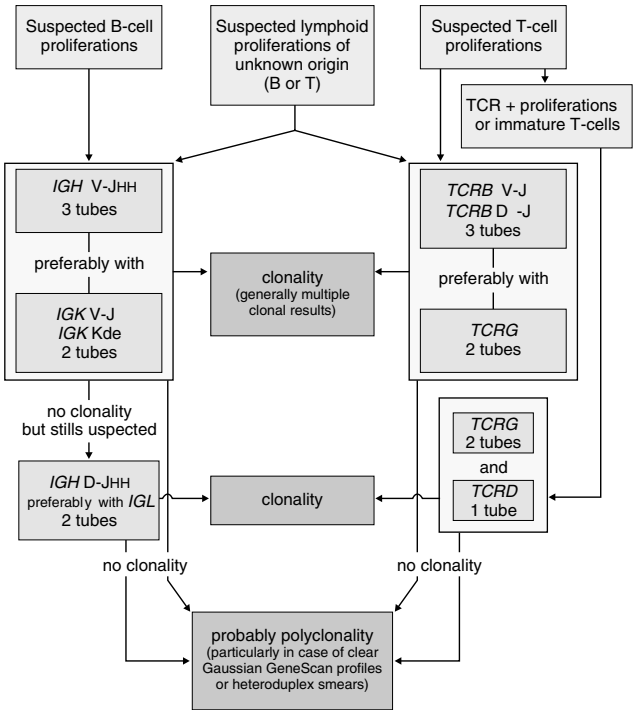

Figure 2 Strategy for PCR-based clonality diagnostics of suspected lymphoproliferations with an inconclusive diagnosis or with unusual histology, immunophenotype or clinical presentation, using the BIOMED-2 multiplex PCR protocols. In case of a suspected B-cell proliferation, firstly *IGH* VH–JH multiplex PCR analysis should be performed, in which the FR1 and FR2 PCR reactions are generally more informative than the FR3 PCR reactions. As a second step, *IGK* PCR analysis (Vκ–Jκ and Kde rearrangements) can be chosen. Preferably, these two steps are combined to avoid delay in the diagnostic process. Finally, *IGH* DH1–6–JH PCR analysis (potentially combined with *IGL* analyses) can be reserved for remaining suspected cases, in which the preceding PCR assays have failed to detect the monoclonality and have not shown clear signs of polyclonality either. For suspected T-cell proliferations, *TCRB* multiplex PCR is generally slightly more informative than *TCRG* PCR, but the order of analysis of these two loci can be changed as they provide complementary information; preferably both targets should be used in parallel. In case of suspected TCR$\gamma\delta^+$ T-cell proliferations and immature T-cell proliferations (suspicion of lymphoblastic malignancies), *TCRG* and *TCRD* PCR analysis is preferred. Because of its complexity, the signal tube *TCRD* assay should not be used in routine T-cell clonality studies. In case of suspected lymphoproliferations of unknown origin, both Ig and TCR genes should be used as PCR targets. It should be noted that in such cases the clonal Ig/TCR results cannot be used straightforwardly for B/T-lineage assignment. A full-proof diagnosis of polyclonality remains difficult, but a high probability of polyclonality is supported by clear Gaussian GeneScan curves or heteroduplex smears in the absence of clonal results.

noted that TCR-gene studies in B-cell malignancies and in reactive tissues frequently show co-existing (small) clonal T-cell populations, presented as weak clonal PCR results. Such results should be interpreted with caution in order to avoid false positivity (see reports by Evans *et al*.[30] and Langerak *et al*.[32]).

Of equal importance in daily practice is the absence of a clonal result and the detection of polyclonal rearrangement patterns in lesions considered to be reactive.[32] In this type of lesions, we discovered clear limitations in the standard pathology diagnosis. Indeed, most of the approximately 100 reactive lesions were polyclonal in nature, but there are a couple of important pitfalls. Most importantly, we did detect a couple of lymphomas missed by experienced hematopathologists.[32] We even discovered a few cases that were disorders that require further characterization, like a case of clonal plasmacytosis in a ruptured spleen (see report by Langerak *et al*.).[32] It is obvious therefore that clonality assessment improves the reliability of lymphoma diagnosis, even in laboratories where specialized hematopathologists perform the diagnostic service.

It should be noted that the above results were obtained with DNA extracted from frozen samples. If formaldehyde-fixed paraffin-embedded tissues are used for DNA extraction, the BIOMED-2 control gene tube should be used to ensure that PCR products of at least 300 bp can be produced.[27] Caution is also needed when small biopsies or biopsies with small tumor infiltrates are processed, such as needle biopsies or extranodal lesions. In such samples, absence of clonal results or the presence of small T-cell clones might lead to incorrect interpretations. Close interaction between the hematopathologist and the molecular biologist is essential in these interpretation steps, for example, to guarantee that the same tissue fraction is used for the histological and molecular analyses and that the results are interpreted accordingly.

In case of unclear results, repeat analyses and consulting of experienced laboratories is preferred over speculation about peak height, surface-under-curve, etc., which we regard as inappropriate usage of multiplex PCR results after 35 cycles or more. Clearly, knowledge and experience is needed for correct interpretation of PCR-based clonality diagnostics. Therefore, the BIOMED-2 Concerted Action is now continued as the Euro-Clonality group for further improvement of the technology and for organization of Clonality Workshops twice per year.

Guideline for efficient clonality testing

We propose a guideline based on our data and their interpretation by experienced hematopathologists and molecular biologists (Figure 2). This guideline needs to be adapted in each laboratory according to the type of pathology samples and the expertise of the pathologist. We do not propose that each tissue specimen suspected of lymphoma should be subjected to the clonality analysis. There are many cases where the diagnosis of lymphoma is straightforward and substantiated by immunohistochemistry, including Ig light chain restriction as a surrogate clonality test. However, we do propose that more cases than until now will be tested in the future, to increase the reliability of the diagnosis to almost 100%. We believe that nowadays this is a service that we need to provide to our patients given the large impact that a wrong diagnosis could have in the clinical setting, treatment for malignant lymphoma has become highly effective, but has serious side effects as well.

Biopsies that are suspected of a malignant lymphoma are subjected to standard histopathological evaluation, followed by a specific panel of antibodies for immunohistochemistry. Whereas in most of these cases a firm diagnosis is obtained, we estimate that about 30% of cases in laboratories with limited specialization in hematopathology and about 10% of cases in specialized hematopathology centers will benefit from clonality testing. We believe that every case with an inconclusive diagnosis and all cases with unusual features in histology, immunophenotype or clinical presentation need to be subjected to clonality testing. Also cases in which the pathological result is in contrast with the clinical findings should benefit from further testing. Clonality testing can be performed in two phases using the most informative targets initially and using a more complete panel of Ig and TCR targets in only a limited number of samples (Figure 2). For reasons of speed and efficiency, it is attractive to use the five indicated Ig tubes and the five indicated TCR tubes

directly in parallel, thereby having the advantage of extra confirmation of clonality based on multiple positive results in the vast majority of malignancies.[30,31]

In principle, Ig genes are used as PCR targets in suspected B-cell proliferations and TCR genes in suspected T-cell proliferations. However, in proliferations of unknown origin both Ig and TCR genes should be analysed in parallel (Figure 2). In such cases, the clonal Ig/TCR gene results should not be used straightforwardly for B/T-lineage assignment, because of the relatively frequent occurrence of cross-lineage Ig/TCR-gene rearrangements,[30,31] particularly in immature lymphoid malignancies.[27]

Conclusion

PCR-based clonality testing in lymphoproliferations has now matured into a reliable method that can easily be used in every laboratory with routine molecular diagnostics. It is important, however, that the test results are interpreted with full knowledge of the immunobiology, Ig/TCR-gene composition and pathology of lymphomas and thus in close cooperation between the molecular biologist and the pathologist. Even though we have not performed a cost-efficiency study, we believe that the relatively low costs of our BIOMED-2 multiprimer, multitarget PCR approach warrants its use in many cases, given the high financial costs and loss of quality of life when a diagnosis of malignant lymphoma is incorrectly made, or alternatively, is not made in an early phase of the disease.

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