Application of BIOMED-2 Primers in Fixed and Decalcified Bone Marrow Biopsies

Analysis of Immunoglobulin H Receptor Rearrangements in B-Cell Non-Hodgkin's Lymphomas

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The detection of clonality in lymphomas was recently improved by the BIOMED-2 approach, but analysis of fixed tissues is limited. Here, we adapted the BIOMED-2 protocol for examining immunoglobulin H (IgH) receptor rearrangements in fixed, decalcified bone marrow biopsies (BMBs) for clonality analysis in B-cell non-Hodgkin's lymphomas (B-NHL). The study included 111 decalcified BMBs (12 formalin fixed and 99 glutardialdehyde fixed), with B-NHL ($n = 85$), T-NHL ($n = 8$), or reactive infiltrates ($n = 18$). Initially, IgH FRIII polymerase chain reaction (PCR) analysis of crude DNA extracts from 75 glutardialdehyde-fixed BMBs (B-NHLs) using a standard seminested PCR resulted in clonal peaks in 46 of 75 (61.3%) BMBs compared with 19 of 70 (27.1%) for the original BIOMED-2 protocol. Modifications to both DNA extraction and PCR reaction improved the detection rate to 26 of 36 (72.2%) for BIOMED-2 primers, including 10 of 15 (66.7%) cases not detected by our standard IgH analysis. Moreover, introducing the same modifications for analysis of the FRII region by BIOMED-2 primers revealed clonal peaks in 19 of 36 (52.8%) B-NHLs compared with 5 of 70 (7.1%) for the original BIOMED-2 protocol. Together, analysis of FRII and FRIII regions by the modified BIOMED-2 protocol increased the detection rate to 31 of 36 (86.1%), particularly for BMBs with histological evidence of follicular lymphoma (FRIII, 70%; FRII, 90%). In summary, this study provides an improved protocol for detection of clonality by IgH-specific BIOMED-2 primers in fixed, decalcified bone marrow biopsies. (*J Mol Diagn* 2005, 7:582–591)

The evaluation of bone marrow samples in patients with non-Hodgkin's lymphoma (NHL) is an important aspect of the diagnosis, especially with respect to staging as well as for assessing treatment response and/or restaging. Besides flow cytometry and fresh bone marrow aspirates, the histopathological classification of fixed, decalcified bone marrow biopsies represents a reliable and standardized approach for such analysis.^{1–3}

Histopathological evaluation of bone marrow biopsies can be supplemented by molecular tools, which may assist subclassification or the diagnosis of suspicious cases. Besides the detection of specific translocations, polymerase chain reaction (PCR) analysis of clonal immunoglobulin H (IgH) and TCR receptor rearrangements is widely used as supplementary tool for the detection of lymphoid malignancies in bone marrow biopsies.^{4–8} However, this requires the integration of molecular data into the histopathological context and can primarily be achieved by using serial sections for morphological and molecular assessment. Moreover, combination of morphologically controlled tissue selection (microdissection) with molecular analysis further increases the specificity and sensitivity of PCR-based clonality assays for small lymphoid infiltrates.^{9,10}

Besides pitfalls introduced by sampling errors or misinterpretation of seemingly "malignant" clonal cell populations,^{11,12} the bias and limitations introduced by technical aspects, especially tissue processing and preservation, are substantial and cannot be corrected for retrospectively. As has been widely anticipated, the fixation of tissues has a major impact on DNA/RNA integrity,^{13–15} and PCR analysis of larger DNA/RNA fragments in such samples is restricted. Although one study reported the successful amplification of DNA with fragment sizes of up to 600 bp,¹⁶ PCR-based amplification of DNA fragments up to 300 bp is generally considered reliable for fixed tissues.¹⁷ Thus, PCR analysis of B-cell clonality in fixed tissues is commonly performed by assays focusing on the FRIII region of the

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Table 1. Summary of Bone Marrow Biopsies Used in the Study

Decalcified bone marrow biopsies (111)					Lymph node
NBF fixed (12)		GA fixed (99)			NBF fixed
Reactive	B-NHL	B-NHL	T-NHL	Reactive	B-NHL
4	8	77	8	14	1

The table summarizes the tissue samples used in the present study, as detailed in Materials and Methods.

IgH chain.^{18–20} These PCR reactions target small DNA segments, readily allowing the amplification of partially degraded DNA and detection of clonal cell populations in about 60 to 80% of formalin-fixed, decalcified bone marrow biopsies.^{17,21}

Although PCR analysis of clonal IgH rearrangements clearly complements histological assessment of non-Hodgkin's lymphomas, the variability of tissues as well as DNA extraction and PCR procedures^{22,23} introduce some limitations to standardization between laboratories. In a recent concerted action within several laboratories of the BIOMED-2 program,^{24,25} standardized protocols for PCR-based clonality analysis in hematopathological malignancies were established for improved detection of B- and T-cell malignancies. Whereas the BIOMED-2 approach was shown to have a high specificity and sensitivity in fresh (frozen) tissue samples, its application to formalin-fixed (nondecalcified) tissue samples was limited, and its use in fixed and decalcified tissues, such as bone marrow biopsies, has not been evaluated so far.

The aim of the present study was to adapt the BIOMED-2 approach for detection of clonal IgH rearrangements in fixed, decalcified bone marrow biopsies and to evaluate its performance compared with a standard seminested IgH PCR. We describe modifications to the original BIOMED-2 protocol that allow its successful application to fixed and decalcified bone marrow biopsies. In a total of 111 BMBs, we show that this modified approach is specific for detection of B-NHLs, irrespective of the type of fixation; is highly sensitive for the detection of B-NHLs with $\geq 5\%$ lymphoid infiltration; and does perform better compared with our standard seminested IgH PCR, particularly in cases with follicular lymphoma.

Materials and Methods

Tissue Samples and Histology

As summarized in Table 1, this study included a total of 111 fixed and decalcified bone marrow biopsies (BMBs) from the archive of the Institute of Pathology in Freiburg. Because our Institute prefers a nonformalin fixative for improved histological evaluation of BMBs, 99 of 111 of the BMBs had been fixed in a combination of 1% buffered formalin, 0.5% glutardialdehyde, and 15.8 g/L calcium-acetate (GA) and only 12 of 111 BMBs had been buffered in 10% neutral buffered formalin (NBF). Irrespective of the type of fixative, all BMBs had been decalcified in 100 g/L EDTA in Tris-hydroxymethyl-amino-methan and had been embedded in paraffin.

All specimens were reclassified histologically before molecular analysis by two experienced pathologists according to the World Health Organization²⁶ and included 85 histologically proven B-NHLs (B-NHL subtypes are given in Results and Table 2), 8 histologically proven T-NHLs, and 18 reactive (reactive lymphocytosis or plasmacytosis) samples. One formalin-fixed (10% buffered formalin) and paraffin-embedded lymph node with a histologically proven B-NHL served as additional positive control.

DNA Extraction

Crude Lysates

Four to eight 10- μ m sections of the selected bone marrow biopsies were pooled in Eppendorf tubes, deparaffinized three times for 10 minutes in Roti-clear (Roth, Karlsruhe, Germany) at 58°C, followed by two times for 10 minutes in 100% ethanol and subsequent lysis in digestion buffer (50 mmol/L Tris-HCl, 1 mmol/L EDTA, 0.5% Tween 20, and 0.5 mg/ μ l proteinase K) at 55°C for 16 hours. The resulting crude lysates were then incubated for 20 minutes at 94°C and stored at 4°C until PCR analysis.

Column Purification

Four serial 5- μ m sections were cut onto glass slides and deparaffinized in xylene (two times for 30 minutes)

Table 2. Performance of the Standard PCR and the Modified BIOMED-2 Approach for IgH Clonality Analysis in B-NHL Subtypes

Lymphoma type	N	Standard IgH FRIII		Modified BIOMED-2 FRIII		Modified BIOMED-2 FRII		Modified BIOMED-2 FRIII and FRII	
		n	%	n	%	n	%	n	%
CLL	8	5	62.5	7	87.5	5	62.5	7	87.5
FL	10	1	10.0	7	70.0	3	30.0	9	90.0
IC	7	6	85.7	5	71.4	4	57.1	7	100.0
MCL	6	4	66.7	5	83.3	4	66.7	5	83.3
Total	31	16	51.6	24	77.4	16	51.6	28	90.3

Results of PCR analysis are compared between B-NHL subtypes for both the standard seminested IgH PCR (FRIII) and the modified BIOMED-2 approach (FRIII/tube C and FRII/tube B). CLL, chronic lymphocytic leukemia; FL, follicular lymphoma; IC, immunocytoma; MCL, mantle cell lymphoma.

and ethanol (two times for 100% and one time for 95, 70, and 50% each for 5 minutes). Subsequently, tissues were scraped off the slides and transferred into Eppendorf tubes, and DNA extractions were performed using the DNeasy kit (Qiagen, Hilden, Germany) following the instructions of the manufacturer. The DNAs were eluted from the column in two steps of each 50- μ l elution buffer and stored at -20°C until PCR analysis.

β -Globin PCR Analysis ("Control" PCR)

Three differently sized fragments of the β -Globin gene (268, 410, and 536 bp) were analyzed by three sets of primers^(27–29) using 10 μ l of DNA. PCR was performed in a reaction volume of 25 μ l, containing 0.4 mmol/L dNTPs, 1 $\times$ AmpliTaq Gold buffer, 2 mmol/L MgCl_2 , and 1 U of AmpliTaq Gold DNA Polymerase (Perkin Elmer, Rodgan, Germany). For the 268-bp amplicon, the PCR was run for 94 $^{\circ}\text{C}$ for 7 minutes, 40 cycles of 94 $^{\circ}\text{C}$ for 1 minute, 55 $^{\circ}\text{C}$ for 1 minute, 72 $^{\circ}\text{C}$ for 1 minute, and a final extension step at 72 $^{\circ}\text{C}$ for 7 minutes. For the 410- and 536-bp amplicons, the PCR was run for 94 $^{\circ}\text{C}$ for 7 minutes, 40 cycles of 94 $^{\circ}\text{C}$ for 1 minute, 60 $^{\circ}\text{C}$ for 1 minute, 72 $^{\circ}\text{C}$ for 2 minutes, and a final extension step at 72 $^{\circ}\text{C}$ for 7 minutes. For assessment of the PCR products, 10 μ l of the PCR products were analyzed on a 2.5% agarose gel, stained by ethidium bromide and visualized using the BioDocAnalyze System (Biometra, Göttingen, Germany).

IgH PCR Analysis

Standard Seminested FRIII PCR

The standard assay for detecting clonal IgH rearrangements was a seminested PCR of the FRIII-J segment.^{18,30} For this, 250 ng of DNA was amplified in a volume of 25 μ l, containing 200 μ mol/L dNTPs (Roth), 1 $\times$ AmpliTaq-buffer (Perkin Elmer), 0.4 μ mol/L of each primer (FR3a, FAM-labeled 5'-ACA CGG CYS TGT ATT ACT GT-3'; and LJH, 5'-TGA GGA GAC GGT GAC C-3') and 1.25 U of AmpliTaq-Gold Polymerase (Perkin Elmer). The PCR was run at 94 $^{\circ}\text{C}$ for 11 minutes followed by 30 cycles of 94 $^{\circ}\text{C}$ for 1 minute, 55 $^{\circ}\text{C}$ for 30 seconds, 72 $^{\circ}\text{C}$ for 30 seconds, and a final extension step at 72 $^{\circ}\text{C}$ for 7 minutes. Two μ l of the PCR products were re-amplified in a reaction volume of 30 μ l containing 200 μ mol/L dNTPs (Roth), 1 $\times$ AmpliTaq buffer (Perkin Elmer), 0.4 μ mol/L each of the FR3a and VLJH primer (5'-GTG ACC AGG GTN CCT TGG CCC CAG-3') and 1.25 U of AmpliTaq-Gold Polymerase (Perkin Elmer). This second PCR was run at 94 $^{\circ}\text{C}$ for 7 minutes followed by 20 cycles of 94 $^{\circ}\text{C}$ for 1 minute, 58 $^{\circ}\text{C}$ for 15 seconds, 72 $^{\circ}\text{C}$ for 30 seconds, and a final extension step at 72 $^{\circ}\text{C}$ for 7 minutes.

Original and Modified BIOMED-2 Protocols

For the original BIOMED-2 protocol for IgH receptor rearrangement analysis, 100 ng of DNA was subjected to

Table 3. Summary of the PCR Protocols for the Original and Modified BIOMED-2 PCR Reactions

	Original BIOMED-2	Modified BIOMED-2
PCR reaction mix		
dNTP	200 μ mol/L	250 μ mol/L
Buffer (MgCl_2)	1.5 mmol/L	2 mmol/L
Primer	0.2 μ mol/L	0.4 μ mol/L
AmpliTaQ Gold	1 U	2.5 U
PCR run parameters		
Cycles	35	40
Denature	45 seconds	1 minute
Anneal	(>) 45 seconds (60 $^{\circ}\text{C}$)	1 minute (60 $^{\circ}\text{C}$)
Extend	1.30 minutes	1 minute

Values of reaction mix contents denote final concentrations, and PCR running parameters are shown for the PTC200 thermal cycler (MJResearch/Biozym, Hess. Oldendorf, Germany).

PCR analysis using FRI/tube A (data not shown), FRII/tube B, and FRIII/tube C primers without any changes to the published protocol.²⁴

Modifications to the BIOMED-2 PCR protocol included changes to the reaction mixtures and cycling conditions, as described in Results and Table 3. Ten microliters of the DNeasy-purified DNA was amplified within a total volume of 50 μ l in 0.5-ml Eppendorf tubes using the PTC200 thermal cycler (MJResearch/Biozym, Hess. Oldendorf, Germany). The reaction mixes were freshly prepared from all reagents during PCR setup (no pre-mixes containing primers and buffers were used).

For assessment of IgH-specific PCR products, genescan analysis of all samples was performed with 1 μ l of PCR product mixed with 10.5 μ l of formamide using a ABI3100 Genetic Analyzer (Applied Biosystems, Weiterstadt, Germany). Analysis of 4 μ l of PCR product did not yield any superior data in preliminary experiments (data not shown). In addition, heteroduplex analysis²⁴ was performed for selected samples (17 of 39). For this, a 6% polyacrylamide gel was prepared and loaded with 15 μ l of the PCR product, and gels were run for 2 hours and stained in 1 mg/ml ethidium bromide for 1 hour. Appropriate size standards were included in genescan and heteroduplex analysis as read-out for fragment length patterns.

Results

Analysis of IgH Rearrangements in Fixed, Decalcified Bone Marrow Samples by Seminested PCR and the BIOMED-2 Protocol

Initially, the performance of the standard seminested PCR and the original BIOMED-2 PCR for IgH-specific clonality analysis was evaluated (Table 4a). The standard seminested PCR of the IgH FRIII region using crude DNA extracts of glutaraldehyde (GA)-fixed, decalcified bone marrow biopsies ($n = 75$) yielded a clonal peak in 46 of 75 (61.3%), oligoclonal peaks in 15 of 75 (20%), and polyclonal peaks in 10 of 75 (13.3%) samples. Amplifica-

Table 4. Comparison of IgH (FRIII region) Clonality Analysis by the Standard Seminested PCR and the BIOMED-2 Approach

(a)	Standard IgH (n = 75)		BIOMED-2c (n = 70)		BIOMED-2b (n = 70)	
	n	%	n	%	n	%
Clonal (small clonal peak)	46	61.3	16 (3)	22.9 (4.2)	5	7.1
Oligoclonal	15	20.0	—	—	—	—
Polyclonal	10	13.3	—	—	—	—
Non-specific	2	2.7	7	10.0	2	2.9
No amplification	2	2.7	44	62.9	63	90

(b)	Standard IgH (n = 36)		BIOMED-2c (n = 31)		BIOMED-2b (n = 31)	
	n	%	n	%	n	%
Clonal (small clonal peak)	21	58.3	7 (1)	22.5 (3.2)	2	6.5
Oligoclonal	6	16.7	—	—	—	—
Polyclonal	6	16.7	—	—	—	—
Non-specific	1	2.8	4	13.0	2	6.5
No amplification	2	5.5	19	61.3	27	87.0

Results of clonality analysis are shown for all GA-fixed, decalcified BMBs with histologically proven B-NHL (a) as well as for the selected representative subset BMBs (n = 36) used for all further experiments (b). Clonal, oligoclonal, or polyclonal from the pattern of genescan analysis (Materials and Methods); non-specific, signal outside the acceptable range of PCR product fragment size.

tion was nonspecific or failed in each 2 of 75 (2.7%) of cases, respectively.

Using the same crude DNA extracts (70 of 75 available) in the BIOMED-2 protocol for the IgH FRIII region (tube C), clonal peaks were observed in 16 of 70 (22.9%) and very weak peaks in an additional of 3 of 70 (4.3%) cases (total, 19 of 70 [27.1%]). The detection rate was not increased by analysis of the IgH FRII region using the BIOMED-2 protocol (tube B), because only 5 of 70 (7.1%) cases exhibited very weak clonal peaks, and these cases had also been positive in the FRIII assay. Finally, amplification of the IgH FRI region by BIOMED-2 primers (tube A) was negative in all cases (data not shown). To examine whether modifications to DNA extraction and/or PCR protocols could improve the detection rates of the BIOMED-2 primers, 36 representative GA-fixed BMBs were selected for further evaluation, as summarized in Table 4(b).

Modifications of DNA Extraction

To evaluate the quality of DNA extracted from GA-fixed, decalcified BMBs, we performed PCR analysis for differentially sized fragments of the β -Globin gene with crude DNA lysates and column-purified DNA extracts from serial sections of the same BMBs (see Materials and Methods).

Reproducible amplification of the 268- and 410-bp fragment was not achieved for the crude DNA extracts (Figure 1a, left panel), with positive signals in 5 of 8 and 1 of 8 samples, respectively. A slight improvement was obtained by further purifying an aliquot of the crude DNA extract via an adapted DNeasy protocol, with positive signals in 8 of 8 and 5 of 8 for the 268- and 410-bp fragment, respectively (Figure 1a, middle panel). However, by using DNeasy-purified DNA extracts from serial BMB sections, reproducible PCR amplification of the 268-bp fragment (11 of 11) as well as the 410-bp fragment (10 of 11) was obtained (Figure 1a, right panel).

Finally, we examined whether the quality of DNeasy-purified DNA extracts differed between formalin- and

GA-fixed, decalcified BMBs using PCR amplification of the 268-bp fragment and a 536-bp fragment of the β -Globin gene. As shown in Figure 1b, there was no significant difference between DNA extracts from differentially fixed BMBs, with reliable amplification of even a 536-bp fragment in each four of five cases.

Comparison of Standard IgH PCR with Crude and DNeasy-Purified DNA Extracts

Having shown that DNeasy-purified DNA performs better in the β -Globin PCR assay, with good amplification of up to 536-bp fragments, we investigated whether this DNA would also improve the results of the standard seminested IgH

Figure 1. Performance of different DNA extracts in PCR analysis of two fragments of the β -Globin gene. **a:** PCR amplification and agarose gel electrophoresis for a 268-bp fragment and 410-bp fragment of the β -Globin gene using 1) crude DNA lysates (**left panel**), 2) crude DNA lysates further column-purified (**middle panel**), and 3) DNeasy-purified DNA extracts (**right panel**). **b:** PCR amplification of the 268-bp fragment and a 536-bp fragment of the β -Globin gene using DNeasy-purified DNA extracts from formalin- and glutaraldehyde-fixed, decalcified bone marrow biopsies.

Table 5. Performance of Different DNA Extracts in the Standard Seminested IgH PCR Assay

Lymphoma type	Crude lysate DNA		DNeasy DNA	
	PCR result	Peak size	PCR result	Peak size
MCL	Clonal	84	Clonal	84
MCL	Clonal	78	Clonal	74
FL	Clonal	70	Clonal	70
FL	Oligoclonal	X	Clonal*	78, 112
CLL	Clonal	65	Clonal	65
CLL	Non-specific	X	Clonal*	80, 83
IC	Non-specific	X	Clonal*	80, 104
IC	Clonal	114	Clonal	112

Results of the standard seminested IgH PCR (Materials and Methods) for crude and DNeasy-purified DNA extracts from serial sections of GA-fixed BMBs with histologically proven B-NHL. PCR reactions were performed on separate days, resulting in a "different" clonal peak in one MCL most likely due to a "stuttering" effect of the Taq polymerase.

*Two clonal peaks were identified in three cases by analysis of DNeasy-purified extracts.

PCR assay. Matching crude and DNeasy-purified DNA extracts from GA-fixed and decalcified BMBs ($n = 8$) with histologically proven B-NHL were amplified by the standard IgH PCR. Clonal peaks were obtained in five of eight cases irrespective of the DNA extraction method, but three additional cases showed clonal peaks on using the DNeasy-purified DNA extracts (Table 5).

Modification of Both DNA Extraction and PCR Protocol Improves IgH Clonality Analysis by BIOMED-2 Primers in Fixed, Decalcified Bone Marrow Biopsies

The DNeasy-purified DNA extracts showed a superior outcome in the housekeeping gene and standard IgH PCR assays. Therefore, this DNA extraction procedure was chosen as basis for further modification of the BIOMED-2 PCR reaction conditions (FRIII/tube C, FRII/tube B), as described in Materials and Methods and Table 3. Because other institutions prefer 10% NBF to GA for fixation of BMBs, both NBF-fixed ($n = 8$) and GA-fixed ($n = 36$, Table 4b) decalcified BMBs, all with histologically proven B-NHL, were included in this series of experiments.

Using DNeasy-purified DNA extracts and the modified BIOMED-2 PCR protocol for FRIII/tube C primers, the detection rate was 6 of 8 (75%) and 26 of 36 (72.2%) for NBF- and GA-fixed BMBs, respectively, with clear single peaks appearing in genescan analysis within the appropriate size range of 100 to 170 bp (Figure 2 and Table 6). Moreover, the use of DNeasy-purified DNA extracts and the modified BIOMED-2 PCR protocol also yielded acceptable results for FRII/tube B primers, with single peaks in 6 of 8 (75%) and 19 of 36 (52.7%) of NBF- and GA-fixed BMBs, respectively (Figure 2 and Table 6).

For NBF-fixed BMBs, matching results for all 6 of 8 (75%) BMBs were obtained for both the FRIII/tube C and FRII/tube B BIOMED-2 assay, and no additional heteroduplex analysis was performed (not shown). For the GA-fixed BMBs, the

overall detection rate of the modified BIOMED-2 assay was 31 of 36 (86.1%), but with 4 of 36 (11.1%) cases yielding a clonal peak exclusively for FRII/tube B, 12 of 36 (33.3%) cases exclusively for FRIII/tube C, and 15 of 36 (41.7%) cases showing a clonal peak in both FRII/tube B and FRIII/tube C (not shown). Therefore, validation of PCR products of 17 GA-fixed BMBs (including cases with clonal, oligoclonal, polyclonal, and negative genescan results) was performed by heteroduplex analysis (Figure 2) and confirmed the genescan results of FRII/tube B (16 of 17, 94%) and FRIII/tube C (14 of 17, 82.4%). Two cases with clonal peaks in genescan analysis had a polyclonal pattern in heteroduplex analysis with a denser smear around the expected PCR product size. The genescan analysis was more sensitive than heteroduplex analysis, because clonal peaks were clearly identifiable when using 1 μ l of PCR product. In contrast, heteroduplex analysis showed only weak bands on the gel, even if 15 μ l of the PCR products was analyzed.

Sensitivity of the Modified BIOMED-2 Approach in Fixed, Decalcified BMBs

First, the sensitivity of IgH clonality analysis was compared between the standard seminested and modified BIOMED-2 approach (Table 7). For this, all investigated B-NHL cases were grouped according to the result of the standard seminested PCR, and the performance of the modified BIOMED-2 approach (FRIII/tube C) was assessed within these subgroups (Table 7, top panel). In total, the modified BIOMED-2 approach yielded additional support for the diagnosis of lymphoma in 10 of 15 cases (66.7%) not detected by the standard assay using the BIOMED-2 FRIII/tube C primers and 13 of 15 (86.7%) cases using both the BIOMED-2 FRIII/tube C and FRII/tube B primers. In contrast, of those cases not detected by the modified BIOMED-2 approach (only FRIII/tube C), standard IgH analysis was positive in 5 of 10 (50%) cases (Table 7, bottom panel).

Second, the sensitivity of the modified BIOMED-2 approach was examined by correlation of the PCR results to the histologically assessed percentage of lymphoid infiltration. For PCR analysis of the IgH FRIII/tube C region, B-NHLs with as few as 5 to 10% of B-NHL-associated lymphoid infiltration were still detectable. In contrast, PCR analysis of the IgH FRII/tube B region only yielded clonal peaks if the B-NHL-associated lymphoid infiltration was at least 20% (Table 8).

Finally, the sensitivity of the modified BIOMED-2 approach was assessed in terms of histological B-NHL subtypes (Table 2) and revealed a major benefit of the modified BIOMED-2 protocol for BMBs with follicular or mantle cell lymphoma compared with the standard seminested PCR (Table 2).

Specificity of the Modified BIOMED-2 Approach in Fixed, Decalcified BMBs

Formalin-fixed and GA-fixed BMBs can readily be analyzed by the modified BIOMED-2 PCR protocol with a high sensitivity, as exemplified by a large series of

Figure 2. Representative results of the modified BIOMED-2 PCR reactions for IgH clonality analysis of the FRII and FRIII region. **Top panels:** Genescan analysis of PCR products obtained from FRII (**left**) and FRIII (**right**) analysis, with fixed and decalcified bone marrow samples with histologically proven B-cell lymphoma (**a–e**), bone marrow sample with a reactive lymphoid infiltrate (**f**), formalin-fixed lymph node sample with histologically proven B-cell lymphoma (**g**), and negative control (**h**; water). **Arrows** indicate clonal peaks within the expected size range. **Bottom panels:** Heteroduplex analysis of the same PCR products (**a–h**) as shown in the top panels. **Left gel:** FRII PCR products; **right gel:** FRIII PCR products.

histologically proven B-NHLs. To examine the specificity of the modified BIOMED-2 protocol, we finally analyzed a series of fixed and decalcified “control” BMBs, including 8 cases with histologically proven T-NHL and 18 cases with reactive lymphocytosis or plasmocytosis and no history of lymphoma. As summarized in Table 9,

Table 6. Comparison of IgH Clonality Analysis between the Modified BIOMED-2 FRIII and FRII Approach

Fixative	N	FRIII only		FRII only		FRIII + FRII		Concordance	
		n	%	n	%	n	%	n	%
Formalin	8	6	75.0	6	75.0	6	75.0	6	75.0
GA	36	26	72.2	19	52.7	31	86.1	14	38.9
Mean	44	34	72.7	25	56.8	37	84.1	20	45.5

Fixed and decalcified bone marrow biopsies with histologically proven B-NHL ($n = 44$) were analyzed using the modified BIOMED-2 protocol for the IgH FRIII and FRII region. The table lists the number and percentage of clonal BMBs detected for only the FRIII, only the FRII, and combined FRIII and FRII analysis. The concordance of BMB detected by both the FRIII and FRII approach is also indicated.

Table 7. Cross-Comparison of the Results from the Standard Seminested and the Modified BIOMED-2 PCR for the IgH FRIII Region

Result of standard IgH (<i>n</i> = 36)		Modified BIOMED-2 (<i>n</i> = 36) clonal peaks		
		FRIII	FRII	FRIII+FRII
Genescan result	<i>N</i>	<i>n</i>	<i>n</i>	
Clonal	21	16	14	18
Oligoclonal	6	4	3	6
Polyclonal	6	4	1	4
Nonspecific	1	0	1	1
No amplification	2	2	0	2

Result of modified BIOMED-2 (<i>n</i> = 36)		Standard IgH (<i>n</i> = 36) clonal peaks	
		FRIII	
Genescan result	<i>N</i>	<i>n</i>	
Clonal	26	16	
Oligoclonal	5	2	
Polyclonal	—	—	
Nonspecific	—	—	
No amplification	5	3	

In the top panel, cases with histological proven B-NHL (*n* = 36) were grouped according to the results of the standard seminested IgH PCR (FRIII region), and within these subgroups, the numbers of positively (clonal) detected cases from the modified BIOMED-2 assay are shown.

In the bottom panel, cases were grouped according to the results of the modified BIOMED-2 assay (FRIII/tube C), and within these subgroups, the numbers of positively (clonal) detected cases by the standard IgH PCR are shown.

clonal peaks were observed in 2 of 8 (25%) T-NHLs (FRIII/tube C) and 1 of 18 (5.6%) of reactive BMBs for each the FRIII/tube C and FRII/tube B PCR reactions.

Discussion

The present study is the first to show that the BIOMED-2 approach for analysis of IgH receptor rearrangements²⁴ can be technically modified to allow its application to formalin- or glutardialdehyde-fixed and decalcified bone marrow biopsies. Moreover, this modified BIOMED-2 protocol clearly improves the detection rate of clonality in bone marrow biopsies with histopathologically proven B-cell non-Hodgkin's lymphomas compared with a standard seminested PCR, especially in cases with follicular lymphoma.

The molecular analysis of IgH receptor rearrangements by conventional PCR and genescan and/or heteroduplex analysis is widely accepted as a supplementary tool to the histopathological evaluation of lymphoma-suspected specimens. A recent improvement of this technique has been introduced by the BIOMED-2 approach.²⁴ However, whereas this assay can be readily performed on fresh tissues or bone marrow aspirates, its application to formalin-fixed tissue specimens is limited.

The aim of the present study was to adapt the BIOMED-2 protocol for analysis of IgH receptor rearrangements in fixed and decalcified bone marrow biopsies and to examine whether this would provide an improved method for detection of B-cell clonality in bone marrow samples compared with our standard seminested PCR assay.

Therefore, we first evaluated the efficiency of our current standard IgH clonality assay, which includes the use of crude DNA extracts and a seminested PCR of the IgH FRIII region.^{18,30} With a positive detection rate of 61.3%,

our approach was well within the detection range published for fixed, decalcified bone marrow biopsies,^{4–8,17,21} particularly because we use a formalin/glutardialdehyde/calcium acetate fixative instead of neutral buffered formalin alone.

In contrast, analysis of the IgH FRIII region by the original BIOMED-2 protocol using the same crude DNA extracts yielded a low detection rate, with strong clonal peaks in only 19 of 70 (27.1%) of cases. This finding was not entirely unexpected because the BIOMED-2 approach is known to be less efficient in formalin-fixed compared with fresh tissue samples²⁴ and the pretreatment of bone marrow biopsies (fixation and decalcification) does introduce another level of complexity for molecular analysis.

However, by introducing an extended de-paraffination and inorganic DNA extraction procedure, we could improve the DNA quality for PCR analysis, as assessed by β -Globin-specific PCR reactions amplifying fragments of up to 536 bp, irrespective of the type of fixative. In fact, the purification of DNA using a column system selects for high molecular weight DNA (>200 bp) and clears DNA extracts from potential PCR inhibitors, which is not achieved by a crude proteinase K-mediated lysis. Moreover, this type of DNA extraction is easily standardized and can be transferred to an automated extraction procedure to facilitate the workflow of molecular pathology laboratories. Therefore, we used column-purified DNA extracts for adaptation of the BIOMED-2 PCR protocols. This was achieved by increasing the amount of MgCl₂ and dNTPs in the reaction mix as well as by increasing the PCR cycling time. These modifications clearly improved the specific detection of clonal IgH rearrangements by BIOMED-2 primers in DNA extracted from fixed, decalcified bone marrow biopsies. Compared with the standard assay, with a detection rate of 61.3%, the mod-

Table 8. Sensitivity of the Modified BIOMED-2 Approach in Terms of Percentage of Lymphoid Infiltration

Infiltration (%)	Standard IgH (FRIII)	Modified BIOMED-2 (FRIII/tube C)	Modified BIOMED-2 (FRII/tube B)
0	Polyclonal	Oligoclonal	x
<5	Clonal	Oligoclonal	Clonal
5	Polyclonal	Clonal	x
10	Polyclonal	Clonal	x
10	X	Clonal	x
10	Clonal	Clonal	x
10	Polyclonal	Oligoclonal	x
15	Oligoclonal	Clonal	x
15	Oligoclonal	Clonal	x
20	Clonal	Clonal	Clonal
20	Polyclonal	Clonal	Clonal
20	Clonal	Clonal	x
20	Clonal	Clonal	x
25	Oligoclonal	Clonal	x
25	Clonal	Clonal	Clonal
25	Clonal	X	x
30	Polyclonal	Clonal	x
35	Clonal	Clonal	Clonal
35	Oligoclonal	x	Clonal
40	Oligoclonal	Clonal	Clonal
50	Clonal	Clonal	Clonal
50	Clonal	Clonal	Clonal
50	Oligoclonal	Oligoclonal	Clonal
55	Clonal	Clonal	Clonal
60	Clonal	Clonal	x
60	x	Clonal	x
60	Clonal	x	x
65	Clonal	Clonal	Clonal
75	Clonal	Clonal	Clonal
80	Clonal	Clonal	Clonal
80	Unspecific	x	Clonal
85	Clonal	Clonal	Clonal
90	Clonal	Clonal	Clonal
95	Clonal	Clonal	Clonal

GA-fixed BMBs with histologically proven B-NHLs were grouped according to the percentage of lymphoid infiltration, and the results of the standard seminested IgH PCR (FRIII) as well as the modified BIOMED-2 approach (FRIII/tube C and FRII/tube B) are listed.

ified BIOMED-2 protocol revealed clonal IgH rearrangements in 72.2% (FRIII/tube C only) and 86.1% (FRIII/tube C and FRII/tube B) of the glutaraldehyde-fixed bone marrow biopsies. In a preliminary test, we have used the same column-purified DNA extracts and PCR modifications to assess the FRI region by BIOMED-2 primers, but only very few cases (2 of 18, 11%) had a prominent clonal peak in the appropriate size range (310–360 bp). For formalin-fixed bone marrow biopsies with histological evidence of B-NHL ($n = 8$), application of the BIOMED-2

FRI/tube A primers with the modified protocol yielded a higher positive detection rate (2 of 8, 25%), but this series was rather small. Therefore, we reason that—similar to the conventional approaches of IgH clonality analysis^{4–8,17,21}—the above-described modified BIOMED-2 protocol for IgH analysis in fixed, decalcified bone marrow samples is most reliable and efficient for the FRII and FRIII region, targeting small fragments of <300 bp.

In the present study, the assessment of the PCR products from the modified BIOMED-2 protocol was done primarily by genescan analysis and was compared with heteroduplex analysis in a subgroup of cases, with matching results in most (tube B, 16 of 17; tube C, 14 of 17) cases. Only cases with a very weak signal in genescan analysis were not detected by heteroduplex analysis. Because of its apparently higher sensitivity, we therefore suggest the use of genescan analysis as primary read-out of the BIOMED-2 PCR products.

The genescan data presented in our study was evaluated strictly according to the suggested fragment length ranges for the individual BIOMED-2 PCR tubes.²⁴ In some cases, genescan analysis revealed clonal peaks about 10 to 40 bp outside the expected range, which we therefore considered as “nonspecific.” However, because we have only included cases with histologically proven B-NHL, further studies should examine whether

Table 9. Specificity of the Modified BIOMED-2 Approach

	N	Modified BIOMED-2 FRIII/tube C		Modified BIOMED-2 FRII/tube B	
		n	%	n	%
NBF					
Reactive infiltrate	4	0	0	0	0
GA					
Reactive infiltrate	14	1	5.6	1	5.6
T-NHL	8	2	25.0	0	0

Fixed and decalcified BMBs without histological evidence of a B-NHL but with T-NHL ($n = 8$) or reactive infiltrates ($n = 18$) were assessed by the modified BIOMED-2 approach, and results are listed for each subgroup.

these nonspecific peaks may represent clonal peaks, for example, by sequencing the PCR products. Moreover, for a routine molecular diagnostic setting, primarily with cases histologically suspicious for lymphoma, we strongly recommend use of duplicate analysis of all samples. Finally, using the modified BIOMED-2 approach, we did reach a high sensitivity irrespective of the type of fixative, with the detection of B-NHLs with as few as 5% lymphoid infiltration (FRIII/tube C).

In addition, the modified BIOMED-2 approach was particularly sensitive for the detection of follicular and mantle cell lymphomas. Moreover, the specificity of the modified BIOMED-2 approach was very high, except two cases with reactive infiltrates and two cases of T-NHL, which showed a clonal IgH rearrangement. For the reactive samples, histological re-examination revealed reactive infiltrates of less than 5%, ie, suggesting that in these cases, the clonal peaks were due to so-called "pseudoclonality." The apparent B-cell "clonality" in the cases with histologically proven T-NHL was also seen by the standard IgH PCR and can be explained by the concept of a cross-lineage rearrangement. Together, these results reinforce the concept of combined histopathological and molecular analysis of bone marrow biopsies, especially the use of serial sections for laboratory analysis and the close interpretation of molecular and histomorphological (clinical) data.

In summary, we describe modifications of the BIOMED-2 PCR protocol for IgH clonality analysis of the FRII and FRIII region (tube B and C) that allow its application to fixed and decalcified bone marrow biopsies. In a large group of histologically proven B-cell lymphomas, this modified assay does have a higher sensitivity for the detection of clonal IgH rearrangements than a standard seminested IgH assay, particularly in cases with follicular lymphoma.

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