

Detection of clonality in follicular lymphoma using formalin-fixed, paraffin-embedded tissue samples and BIOMED-2 immunoglobulin primers

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ABSTRACT

Aims The BIOMED-2 multiplex PCR protocol is a commonly used procedure for assessing B cell clonality in lymphoma diagnostics. Follicular lymphoma poses a special challenge for PCR-based analyses because of high prevalence of somatic hypermutations in the rearranged immunoglobulin (IG) domains. This study aimed to evaluate the BIOMED-2 protocol performance in detection of B cell clonality in follicular lymphoma using formalin-fixed, paraffin-embedded (FFPE) tissue.

Methods FFPE samples from 118 patients diagnosed with follicular lymphoma in the period 1998–2008 were used in the study. Clonality of IG heavy (*IGH*) and light chains (*IGK*, *IGL*) was assessed using a PCR procedure that was optimised for FFPE tissue.

Results The highest clonal detection rates were 67.8% with the *IGH* V_H-FR2-J_H assay and 66.1% with the *IGK* V_K-J_K assay. Clonality was detected in 94.9% of all FFPE follicular lymphoma samples when all assays were combined. FFPE samples stored for 1–5 years did not perform significantly differently from those stored for 6–11 years. Interobserver agreement of clonality was tested for all analyses. The lowest score (Cohen's κ value = 0.56) was observed for the *IGK* V_K-J_K clonality assay.

Conclusions An improved PCR protocol for detection of clonality in FFPE samples using BIOMED-2 IG primers is presented. For best performance, a combination of *IGH* and *IGK* analyses is recommended.

INTRODUCTION

PCR assays for immunoglobulin (IG) gene rearrangements are used to distinguish reactive from malignant B cell proliferations in cases that are difficult to assess morphologically and immunophenotypically.^{1–2} Many different PCR strategies for IG clonality testing have been described.^{3–9} Standardised PCR assays, developed by the BIOMED-2 group, are now available for the analysis of complete and incomplete *IGH*, as well as *IGK* and *IGL*, gene rearrangements.¹⁰

The BIOMED-2 assays have been extensively validated for fresh and frozen tissues.^{10–13} However, FFPE samples constitute the majority of diagnostic biopsies submitted for analysis in most pathology laboratories. The fixation and storage of tissues has a well-known impact on DNA integrity.^{14–15} Generally, DNA fragments up to 200–300 base pairs (bp) can be reliably amplified from FFPE tissues.^{16–17} Although the BIOMED-2 primers were designed so that the PCR products would be <300 bp preferably, they have been found to be less

efficient in the few FFPE specimens that have been tested.¹⁰

One important limitation in evaluation of rearranged IG genes by PCR is related to the frequency of somatic hypermutations arising in stimulated germinal centre B cells. Such mutations reduce binding efficiency of the complete *IGH* rearrangement primers. Hence, the detection of clonality by IG clonality assays in postgerminal centre B cell malignancies, such as in follicular lymphomas, is more challenging than in other lymphoma types.^{10–11–18}

The aim of the present study was to evaluate the performance of the BIOMED-2 IG primers in FFPE samples of follicular lymphoma. We also describe modifications of the original BIOMED-2 protocol that allow more successful application for FFPE biopsies.

MATERIALS AND METHODS

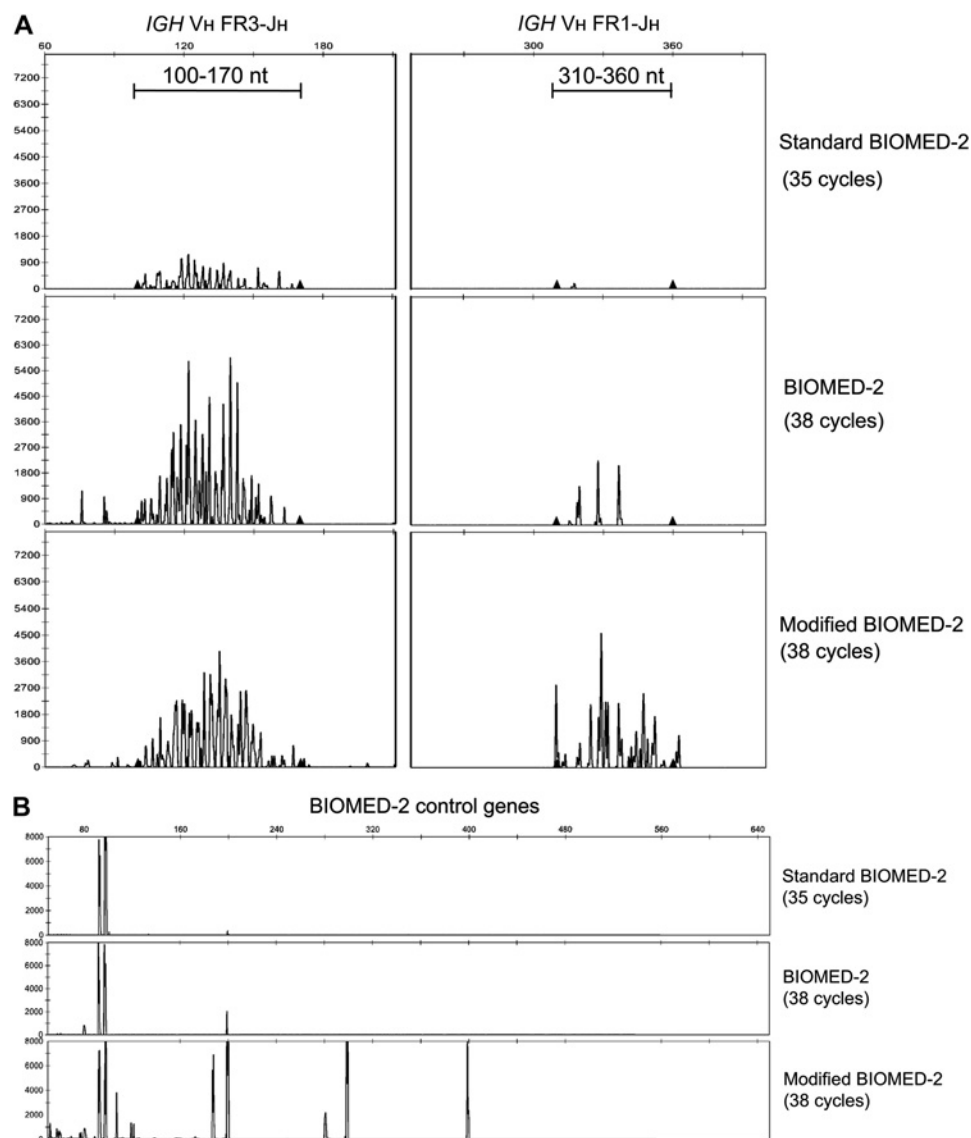
Tissue specimens

One FFPE tissue block from each of 118 patients diagnosed with follicular lymphoma in the period 1998–2008 was obtained from the archives of the Department of Pathology, Haukeland University Hospital, Bergen, Norway. Sixty-two (53%) of the specimens were from 1998 to 2003, and the remaining were from 2004 to 2008. The specimens were biopsies or surgical resections from lymph nodes (n=102) and extranodal sites (gastrointestinal tract (n=7), tonsils (2), salivary glands (2), ovary (1), uterus (1), ocular adnexa (1), brain (1) and skin (1)). Before molecular analyses, all cases were re-evaluated by a haematopathologist (LH) employing the WHO classification of lymphoid neoplasms.¹⁹ Among the cases, 49 were grade 1, 42 were grade 2, 20 were grade 3A, and 7 were grade 3B. Areas of diffuse large B cell lymphoma were observed in nine of the grade 3 lymphomas. No cases with partial nodal involvement or in situ follicular lymphoma were included. FFPE samples from 10 reactive tonsils were included as control tissue.

DNA extraction

Two 10 μ m sections were cut from each paraffin block. The sections were deparaffinised with xylene and dehydrated in 100% ethanol, before overnight proteinase K (20 mg/ml) digestion. An automated workstation (BioRobot M48; Qiagen, Hilden, Germany) was used for DNA extraction. DNA concentration and quality of DNA were determined by spectrophotometry (ND1000; NanoDrop Technologies, Wilmington, Delaware, USA). Average

Figure 1 (A) Polyclonal curves for the *IGH* V_H-FR3-J_H (left) assay and the V_H-FR1-J_H (right) assay using the standard BIOMED-2 protocol, the BIOMED-2 protocol with 38 PCR cycles and a modified BIOMED-2 protocol (38 PCR cycles and a commercial multiplex PCR kit); nt, nucleotide. (B) Example of the three protocols applied on the set of BIOMED-2 control genes. The same sample was used for each protocol.



DNA concentration, OD260/280 and OD260/230 ratios were 73 ng/μl (range 6–207 ng/μl), 1.79 and 1.50, respectively. The quality of each DNA sample was also analysed by the BIOMED-2 PCR control genes.¹⁰

Multiplex PCR amplification

The clonality analyses were adapted for archival tissue by using the Multiplex PCR kit (catalogue no. 206143; Qiagen) with HotStarTaq DNA polymerase. All eight BIOMED-2 IG primer sets were run separately.¹⁰ All reverse primers were labelled with fluorescent dye (6-FAM). The modified BIOMED-2 protocol was carried out by mixing 2.5 μl primer mix (2 μM of each primer), 12.5 μl of the Multiplex buffer and 2.5 μl DNA template to a final reaction volume of 25 μl by adding H₂O. PCR conditions were as follows: an initial activation step (95°C, 15 min), 38 cycles consisting of denaturation (95°C, 45 s), annealing of PCR primers (60°C, 90 s) and extension (72°C, 90 s), and a final extension step (72°C for 10 min, followed by cooling to 4°C). All samples were run with undiluted and fivefold-diluted template DNA. Polyclonal and non-template controls were always included. The PCR products were subjected to fragment analysis on an ABI 3100 capillary sequencer (Applied Biosystems, Foster City, California, USA).

Assessment of clonality and interobserver agreement

Identification of one or two definite and consistent PCR peak(s) by fragment analysis within the expected size range was interpreted as positive for clonality. Discrete peaks that were located in the centre of the expected size range, or were associated with a polyclonal background, were interpreted as polyclonal. Some samples yielded few amplified fragments (number <3–4),

Table 1 Clonal rearrangement detected in formalin-fixed, paraffin-embedded follicular lymphomas, and level of agreement estimated by Cohen's κ

PCR assay	Clonality detection rate (%), n = 118	Cohen's κ
<i>IGH</i> V _H -FR1-J _H	57.6	0.93
<i>IGH</i> V _H -FR2-J _H	67.8	0.89
<i>IGH</i> V _H -FR3-J _H	38.1	0.91
<i>IGH</i> D _H -J _H	8.5	0.84
<i>IGH</i> D _H 7-J _H	0	ND
<i>IGK</i> V _K -J _K	66.1	0.56
<i>IGK</i> V _K /intron-Kde	50.0	0.88
<i>IGL</i> V _L -J _L	8.5	0.73

ND, not determined.

Table 2 The combined use of complete *IGH* and *IGK* rearrangement assays improves clonality detection in formalin-fixed, paraffin-embedded follicular lymphoma samples

Combined BIOMED-2 PCR assays		No. of assays	Clonality detection rate (%), n=118	Samples with a single clonal result (%), n=118
<i>IGH</i>	V _H -FR1-J _H , V _H -FR2-J _H , V _H -FR3-J _H	3	79.7	19.5
<i>IGK</i>	V _K -J _K , V _K /intron-Kde	2	82.2	48.3
<i>IGH</i> + <i>IGK</i>	V _H -FR2-J _H , V _K -J _K	2	86.4	39.0
<i>IGH</i> + <i>IGK</i>	V _H -FR2-J _H , V _H -FR3-J _H , V _K -J _K	3	91.5	31.4
<i>IGH</i> + <i>IGK</i>	V _H -FR2-J _H , V _H -FR3-J _H , V _K -J _K , V _K /intron-Kde	4	94.9	20.3
<i>IGH</i> + <i>IGK</i>	V _H -FR1-J _H , V _H -FR2-J _H , V _H -FR3-J _H , V _K -J _K , V _K /intron-Kde	5	94.9	15.3

Stepwise combinations of complete *IGH* and *IGK* rearrangement assays are shown in rows 3–6. Only data for the best combination per number of assays (2, 3, 4 and 5) are shown.

fragments of low intensity (products <2000 fluorescent units), or even no amplified products. These were regarded as low-intensity curves, and were classified as polyclonal for the purpose of this study.

As previously described,^{2, 10} peaks just outside the 5–95% size ranges were accepted as true rearrangement products. Two samples showed peaks of approximately 234 bp using the *IGH* V_H-FR2-J_H assay, and one sample showed a peak of approximately 94 bp using the *IGH* V_H-FR3-J_H assay. These peaks were considered undersized, and the samples were sequenced for evidence of clonality.

All samples were assessed for clonality by two independent raters (EB, OKV) before a consensus was established. Agreement between raters was expressed using Cohen's κ coefficient.²⁰ A κ value of 1 indicated perfect agreement, whereas a κ value of 0 indicated no agreement.²¹

Statistical analysis

The χ^2 test was used to compare frequencies. Where applicable, Fisher's exact test was performed to compare two categorical variables. A significant finding was defined as a *p* value <0.05. All statistical analysis was carried out using the statistical package SPSS Statistics 17.0 (SPSS, Chicago, Illinois, USA).

RESULTS

Evaluation of clonality

In a pilot study, three lymphoma samples were analysed with the aim of optimising PCR conditions for FFPE tissue. Three various protocols were tested: (1) the standard BIOMED-2 procedure, which includes 35 PCR cycles¹⁰; (2) the standard BIOMED-2 procedure with the number of PCR cycles increased to 38; and (3) a modified BIOMED-2 procedure using a commercial multiplex PCR kit and 38 PCR cycles (see Materials and methods). For assays with amplified fragments <250 bp, such as the *IGH* V_H-FR3-J_H assay, good performance with respect to obtaining a smooth polyclonal profile was achieved by increasing the number of PCR cycles (figure 1A). However, for assays with larger amplified fragments, the modified BIOMED-2 protocol was superior.

All DNA samples were also tested using the BIOMED-2 control genes. PCR products of >300 bp were observed in 11 samples (9.3%) using the standard BIOMED-2 procedure, in 56 samples (47.5%) when the number of PCR cycles were increased to 38, and in 112 samples (94.4%) when the modified BIOMED-2 protocol was used (figure 1B). The modified BIOMED-2 protocol was thereafter employed for assessment of clonality throughout the study.

The clonality detection rate obtained by each assay in all FFPE samples (n=118) is shown in table 1. Six cases did not show any clonal rearrangements; supplementary fluorescence in situ hybridisation analysis (BCL2 and BCL6 split-apart approach) revealed BCL2 translocation in four of these cases, BCL2 amplification in one, and one was negative (data not shown).

Determination of clonality requires experience and cannot be assessed by fully objective means. The level of agreement was therefore tested on all assays (table 1). Cohen's κ value was lowest for the *IGK* V_K-J_K assay (0.56).

The combined use of three complete *IGH* rearrangement assays established clonality in 94 of the 118 (79.7%) cases (table 2). When the *IGK* assays (V_K-J_K and V_K/intron-Kde) were used, clonality was found in 97 (82.2%) cases. By using a combination of the three complete *IGH* rearrangement assays and the two *IGK* assays, clonality was achieved in 94.9% of the samples. Of note, the incomplete *IGH* assays (D_H-J_H and D_H7-J_H) and the *IGL* assay (V_λ-J_λ) did not identify any clonal result that was not identified by the other assays.

Effect of age and DNA concentration

Clonality detection using the three complete *IGH* rearrangement assays was compared on blocks from the periods 1998–2003 and 2004–2008. There was a tendency to a higher rate of clonality detection on blocks from 2004 to 2008 than on blocks from 1998 to 2003 for the V_H-FR1-J_H and V_H-FR2-J_H assays, but not for the V_H-FR3-J_H assay (table 3).

For the V_H-FR1-J_H and V_H-FR2-J_H assays, the amount of DNA did not have a significant effect on detection of clonality on the 2004–2008 samples (table 3). However, among the 1998–2003 samples, we noted a somewhat lower detection rate for those

Table 3 Clonality detection rate by the complete *IGH* rearrangement assays according to DNA sample concentration and period stored

DNA (ng/μl)	n	% Clonality V _H -FR1-J _H			% Clonality V _H -FR2-J _H			% Clonality V _H -FR3-J _H		
		1998–2003	2004–2008	All samples	1998–2003	2004–2008	All samples	1998–2003	2004–2008	All samples
<50	45	37.5 (6/19)	65.4 (17/26)	51.1 (23/45)	57.9 (11/19)	69.2 (18/26)	64.4 (29/45)	36.8 (7/19)	23.1 (6/26)	28.9 (13/45)
50–100	40	57.1 (12/21)	63.1 (12/19)	60.0 (24/40)	66.7 (14/21)	68.4 (13/19)	67.5 (27/40)	57.1 (12/21)	36.8 (7/19)	47.5 (19/40)
>100	33	63.6 (14/22)	63.6 (7/11)	63.6 (21/33)	72.7 (16/22)	72.7 (8/11)	72.7 (24/33)	45.5 (10/22)	27.2 (3/11)	39.3 (13/33)
Total	118	51.6 (32/62)	64.3 (36/56)	57.6 (68/118)	66.1 (41/62)	69.6 (39/56)	67.8 (80/118)	46.8 (29/62)	28.6 (16/56)	38.1 (45/118)

Table 4 Frequency of low-intensity curves in the complete *IGH* and *IGK* rearrangements assays according to DNA sample concentration

DNA (ng/μl)	n	Frequency of low-intensity curves (%)				
		VH-FR1-JH	VH-FR2-JH	VH-FR3-JH	Vκ-Jκ	Vκ/intron-Kde
<50	45	28.9	11.1	4.4	2.2	46.7
50–100	40	17.5	12.5	2.5	2.5	30.0
>100	33	15.2	9.1	3.0	3.0	27.3
Total	118	21.2	11.0	3.4	2.5	35.6

samples with the lowest (<50 ng/μl) concentration of DNA. On the other hand, the detection of clonality in the VH-FR3-JH assay appeared not to be negatively affected by storage or a low DNA content.

Also the occurrence of low-intensity curves was evaluated in relation to storage time and DNA content for the three complete *IGH* rearrangement assays and the two *IGK* assays (table 4). Increased numbers of low-intensity curves were found for the *IGH* VH-FR1-JH and the *IGK* Vκ/intron-Kde assays ($p<0.001$) as compared with a PCR assay with shorter amplified fragments, such as *IGH* VH-FR2-JH. It was also observed that the numbers of such low-intensity curves were highest for samples with DNA concentration <50 ng/μl.

DISCUSSION

The performance of the BIOMED-2 IG primers on routine diagnostic FFPE tissue and more specifically on follicular lymphomas has not been extensively investigated. Only a small number of samples or a limited number of analyses have been reported on.^{22–25}

In our study, the clonality detection rate in FFPE follicular lymphoma was somewhat lower than previously reported for fresh/frozen follicular lymphoma samples.^{10 11 13 26} Only the *IGK* Vκ-Jκ assay detected a higher rate of clonality (66%) in our study than the rate reported (63%) for fresh/frozen samples.¹¹ In addition, the Vκ-Jκ assay differed from the other BIOMED-2 assays with lower interobserver agreement by Cohen's κ (0.56) (table 1). The difficulty in clonality interpretation of the Vκ-Jκ assay can be explained by the restricted CDR3 region in these rearrangements, and it has also been noticed earlier by McClure *et al*²³ and Evans *et al*.¹¹

Used in combination, the detection rates for the three complete *IGH* and the two *IGK* assays were 79.7% and 82.2%, respectively (table 2). Previous studies on FFPE follicular lymphomas have reported lower detection rate (range 35–55%) for the complete BIOMED-2 *IGH* assays.^{22 23 27} An improved clonality detection rate was also observed as compared with previously used primer systems.^{3 28–30} The clonality detection rate was further improved by combining the three complete *IGH* and the two *IGK* rearrangements assays, and a clonal result was obtained in 94.9% of the cases.

To reflect the broad range of DNA concentrations found in diagnostic samples, we did not attempt to standardise the DNA concentration. The samples were instead divided into three DNA concentration groups. In particular, for assays with amplified fragments >250 bp there was a tendency of a higher clonal yield with increasing amount of DNA (table 3). There was also a tendency to higher clonality detection rates in the samples from 2004 to 2008, as compared with the samples from 1998 to 2003 when DNA concentrations were <100 ng/μl. However, there was no statistical difference in these assays in the rate of clonality observed in the samples from 2004 to 2008 as compared with older samples. For some other assays, such as

Take-home messages

- ▶ A modified BIOMED-2 PCR protocol showed improved performance for detection of clonality in follicular lymphomas on formalin-fixed, paraffin-embedded tissue samples.
- ▶ For diagnostic purposes, a combination of *IGH* and *IGK* analysis was recommended.
- ▶ Aged versus more recently obtained formalin-fixed, paraffin-embedded samples did not perform significantly different with respect to detection of clonality.

the *IGH* VH-FR3-JH, a DNA content <100 ng/μl performed better than a higher DNA content. The reason for this latter observation is not clear.

It has also been noted that false-negative clonality outcomes and amplification failure are more frequent in FFPE tissue than in fresh/frozen follicular lymphoma samples.^{25 31} In our study, some degree of amplification failure was observed in assays with PCR products >250–300 bp such as the *IGH* VH-FR1-JH and the *IGK* Vκ/intron-Kde assays, and especially for samples with DNA concentrations <50 ng/μl (table 4). The problem of low-intensity curves does need close attention. Our results did, however, indicate that the problem was not significant enough to advocate a split diagnostic protocol to ensure a higher yield by PCR in those cases. The main reason for this was that we observed a high detection rate of clonality and that a panel of clonality assays should always be used for diagnostic purposes.

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Competing interests None to declare.

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