A practical strategy for the routine use of BIOMED-2 PCR assays for detection of B- and T-cell clonality in diagnostic haematopathology

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Summary
BIOMED-2 polymerase chain reaction (PCR) assays for clonality analysis of immunoglobulin (IG) and T-cell receptor (TCR) gene rearrangements were evaluated in routine haematopathological practice where paraffin-embedded tissues constitute the majority of specimens. One hundred and twenty-five fresh/frozen and 316 paraffin specimens were analysed for DNA quality and clonality. Seventy-nine per cent of paraffin specimens yielded PCR products of over 300 bp. These specimens and all fresh/frozen specimens were analysed with the complete set of BIOMED-2 reactions for IG (8 reactions) and/or TCR (6 reactions) gene rearrangements. The rate of detection of clonality was 96% in mature B-cell neoplasms and 98% in mature T-cell neoplasms and there were no significant differences in these rates between paraffin and fresh/frozen specimens. As the value of sole use of any individual BIOMED-2 reaction in clonality detection was limited, we assessed combinations of reactions that gave the greatest sensitivity with fewest reactions and were applicable for both fresh/frozen and paraffin specimens. For IG gene rearrangements, three reactions combining one targeting the IG heavy chain framework-2 region and two targeting the IG kappa locus achieved a 91% detection rate. For TCR gene rearrangements, the two TCR gamma reactions gave a 94% detection rate. We therefore recommend this strategy as the first-line assays for routine B- and T-cell clonality analysis in diagnostic haematopathology.

Keywords: BIOMED-2, PCR clonality analysis, lymphoma, paraffin-embedded specimens, haematopathology.
thermocycling programme followed by heteroduplex treatment and polyacrylamide gel electrophoresis (PAGE) or GeneScan analysis of the PCR products. Following validation on 90 Southern-blot defined DNA samples from various types of World Health Organisation (WHO)-defined lymphoid malignancies, these assays were found to be highly sensitive and effective in the detection of clonal IG and TCR gene rearrangements and were recommended as the standard methods for diagnostic clonality studies in suspected LPD.

However, the BIOMED-2 PCR assays were not evaluated for use in routine haematopathological practice where paraffin-embedded tissues constitute the majority of specimens. Fixation of tissues causes extensive fragmentation of nucleotides, thus adversely affecting the subsequent PCR analysis (Greer et al., 1991; Liu et al., 2002). Although BIOMED-2 primers were designed to limit the size of most PCR products to less than 300 bp for use with paraffin-embedded tissues, they were only tested in a small number of paraffin specimens and were found to be less efficient than when used with fresh tissues (van Dongen et al., 2003). Furthermore, as the number of multiplexed BIOMED-2 reactions developed for complete coverage of IG (eight reactions) and TCR (six reactions) gene rearrangements is relatively large and the spectra of gene rearrangements detected by different primer sets show considerable overlap, it is not practical, and perhaps not necessary, to perform all these reactions on every case submitted for clonality analysis. The establishment of a practical strategy for application of BIOMED-2 assays to achieve the highest rate of detection of clonality with the minimum number of reactions would therefore be valuable for routine diagnostic haematopathology. Although such a strategy was discussed by the original BIOMED-2 study, data on the rate of detection of clonality by each individual reaction is not available and the best combinations of these reactions have not been systematically assessed.

The present study was initiated shortly after the publication of the BIOMED-2 primers and protocols. We applied BIOMED-2 PCR assays to a series of 441 specimens, including 316 paraffin-embedded specimens, referred consecutively for clonality analysis during a 2-year period (i) to determine their clinical sensitivity and specificity for the detection of clonality in routine haematopathological specimens, especially paraffin specimens; (ii) to evaluate the efficacy of each individual BIOMED-2 reaction and various combinations of these reactions in detecting clonal IG and TCR gene rearrangements; and (iii) to develop a practical strategy for the application of BIOMED-2 PCR assays in routine diagnostic clonality analysis.

During the preparation of this manuscript, the participants of BIOMED-2 reported large-scale follow-up studies of the BIOMED-2 assays in B-cell malignancies, T-cell malignancies and reactive lesions (Bruggemann et al., 2007; Evans et al., 2007; Langerak et al., 2007). These studies validated the efficiency of the BIOMED-2 assays and provided a guideline for their use in routine clonality testing (van Krieken et al., 2007). However, these studies used only selected fresh or frozen samples with abundant good quality material. Application of the BIOMED-2 PCR protocols to routine paraffin specimens, which vary considerably in quality and are often too small for DNA purification, remains to be further evaluated. An efficient strategy for application of the many BIOMED-2 reactions to routine histological specimens also remains to be determined. Our present work addresses these issues and is thus complementary to the studies of the BIOMED-2 consortium. We demonstrate that the BIOMED-2 protocols are highly efficient in routine paraffin specimens provided that the DNA extracted can be amplified for PCR products of over 300 bp. We also provide a strategy for clonality analysis that is an efficient refinement of the BIOMED-2 strategy and equally suitable for routine fresh and paraffin samples.

Materials and methods

Specimens and patients

A total of 441 consecutive specimens from 395 patients with a suspected mature LPD were submitted for clonality analysis of IG or TCR gene rearrangements, or both, as part of a diagnostic work-up during a 2-year period from 2003 to 2005. These included 125 fresh/frozen specimens (125 patients) and 316 paraffin-embedded specimens (278 patients). The fresh/frozen specimens were mainly bone marrow (BM) aspirates (83, 66%) and peripheral blood (36, 29%) whereas the paraffin specimens were composed of biopsies or surgical resections of various tissues but were mostly from lymph node (114, 36%), gastrointestinal tract (83, 26%) and skin (39, 12%) (Table SI). In total, 362 specimens were for primary diagnosis, 20 were for staging and 59 were for follow-up. Forty-one patients had multiple specimens. The local research ethics committee approved the use of these pathological materials for research.

DNA preparation

DNA was prepared from most paraffin specimens as a crude tissue digest. Sections of 5 μm thickness were cut from each paraffin block or, in cases where unstained paraffin sections were the only material available, they were scraped off slides into a microcentrifuge tube. The tissue was then deparaffinised with xylene, washed twice with 100% ethanol and digested, depending on the size of the tissue, in 50–200 μl solution containing 100 mmol/l Tris-HCl, pH 8.3, 500 mmol/l KCl, 0.5% triton and 200 μg/ml protease K (Qiagen, West Sussex, UK) at 56°C overnight. The crude extract was then heated to 95°C for 10 min and stored at −20°C until PCR analysis.

For paraffin preparations of larger surgical resections, tissues with extensive haemorrhage or necrosis, liver and fresh/frozen tissues, DNA was extracted and purified using a QIAamp® DNA Mini Kit (Qiagen). For fresh peripheral blood, BM aspirates and cytological specimens, DNA was prepared using
the Nucleon® Genomic DNA Extraction kit (Tepnel Life Sciences, Manchester, UK).

**PCR analysis**

PCR amplifications were performed using commercial BIOMED-2 multiplex PCR master mixes and controls (InVivoScribe Technologies, San Diego, CA, USA) and the BIOMED-2 PCR protocols (van Dongen et al., 2003) with the following minor modifications. Each PCR was carried out in a 25 μl volume in duplicate. For purified DNA samples, 50–200 ng of DNA was used in each PCR. For crude tissue digests, 1 and 2 μl of the supernatant were used in two parallel PCR amplifications. In these cases, if PCR amplification was poor or not reproducible due to too little or too much DNA template, the PCR was repeated with the amount of template DNA adjusted accordingly.

Following assessment of DNA quality using the BIOMED-2 specimen control reaction, all samples yielding control gene PCR products of over 300 bp were subjected to the full set of BIOMED-2 reactions for IG gene arrangements if B-cell proliferation was suspected, the full set of BIOMED-2 reactions for TCR gene rearrangements if T-cell proliferation was suspected, or both if the lineage of the lymphoid infiltrate was unclear. The full set of reactions for IG gene arrangements included five reactions targeting IGH (IGH<sub>A</sub>, IGH<sub>B</sub>, IGH<sub>C</sub>, IGH<sub>D</sub>, IGH<sub>E</sub>, IGH<sub>F</sub>), two reactions targeting IGK (IGH<sub>J</sub>), two reactions targeting IGL (IGH<sub>K</sub>), and one reaction for IGL (IGH<sub>L</sub>). The full set of reactions for TCR gene arrangements included two reactions targeting TCRG (TCRG<sub>A</sub>, TCRG<sub>B</sub>, TCRG<sub>C</sub>), three reactions targeting TCRB (TCRB<sub>A</sub>, TCRB<sub>B</sub>, TCRB<sub>C</sub>), and one reaction targeting TCRD (TCRD<sub>A</sub>). Each reaction was carried out in a 25 μl volume. For PCR products greater than 300 bp, of which 223 (89%) produced products greater than 400 bp. As the majority of BIOMED-2 PCR primers were designed to generate products of less than 300 bp (van Dongen et al., 2003), all samples yielding control PCR products greater than 300 bp were considered to be suitable for clonality analysis.

**Diagnosis of LPD**

All specimens were subjected to a comprehensive morphological and immunophenotypic examination during routine diagnostic assessment. Those with an atypical lymphoid proliferation suspicious of malignancy and those considered neoplastic but in need of molecular genetic evidence of malignancy were referred to our laboratories for molecular analysis. In addition to clonality analysis, both PCR and cytogenetic analysis (including interphase fluorescence in situ hybridisation, FISH) for detection of chromosomal translocations, and in situ hybridisation to detect small non-coding RNAs of Epstein–Barr virus (EBV) were performed as required to aid diagnosis and classification. The final diagnosis was made based on all available clinical, morphological, immunophenotypic and molecular genetic information. The lymphomas were classified according to the WHO Classification of Tumours of Hematopoietic and Lymphoid Tissues (Jaffe et al., 2001) and the WHO-European Organisation for Research and Treatment of Cancer (EORTC) classification for cutaneous lymphomas (Willenmez et al., 2005). A specialist haematopathologist or haematologist reviewed the diagnosis of each case.

**Statistical analysis**

Where applicable, Fisher’s exact test was performed to compare two categorical variables. Differences were considered significant when P-values were less than 0.05.

**Results**

**Quality of specimens**

The DNA prepared from each of 125 fresh/frozen specimens was of high yield and quality. Of 316 paraffin specimens analysed, 251 (79%) produced control PCR products of greater than 300 bp, of which 223 (89%) produced products greater than 400 bp. As the majority of BIOMED-2 PCR primers were designed to generate products of less than 300 bp (van Dongen et al., 2003), all samples yielding control PCR products greater than 300 bp were considered to be suitable for clonality analysis.
with BIOMED-2 assays. The 65 paraffin specimens with PCR products of less than 300 bp were excluded from further study. Overall, 281 specimens (91 fresh/frozen and 190 paraffin) were analysed with the full set of BIOMED-2 reactions for IG gene rearrangements and 144 specimens (47 fresh/frozen and 97 paraffin) were analysed with the full set of BIOMED-2 reactions for TCR gene arrangements. Of these, 49 specimens (13 fresh/frozen and 36 paraffin) were analysed for both (Table SI).

Detection of clonal IG gene rearrangements in mature B-cell LPD

Sensitivity and specificity. Using the full set of BIOMED-2 reactions for B-cell clonality, clonal IG gene rearrangements were detected in 147 of 153 (96%) specimens diagnosed with a mature B-cell neoplasm (49 of 51 fresh/frozen (96%) and 98 of 102 paraffin (96%) specimens). In contrast, clonal IG gene rearrangements were not detected in any of the 70 diagnostic specimens without evidence of B-cell neoplasia (16 fresh/frozen and 54 paraffin specimens) (Table I). Detection of clonal IG gene rearrangements using the BIOMED-2 PCR assays was therefore highly sensitive and specific and there was no difference between paraffin and fresh/frozen specimens in these respects as long as the DNA quality was adequate for the clonality analysis.

The six cases with mature B-cell neoplasms for which no clonal IG gene rearrangement could be detected included one unclassified B-cell non-Hodgkin lymphoma (B-NHL), one myeloma, one salivary gland mucosa-associated lymphoid tissue (MALT) lymphoma, one nodal diffuse large B-cell lymphoma (DLBCL) and two nodal follicular lymphomas (FL). The specimen with unclassified B-NHL was a haemodilute staging BM aspirate without evidence of light chain restriction by flow cytometry. However, the corresponding BM trephine showed an atypical lymphoid infiltrate. The inability to detect a clonal IG gene rearrangement was most likely a result of inadequate BM aspirate sampling. The diagnosis of the other five cases was supported by immunophenotyping and FISH study of chromosomal translocations. Failure to detect clonal IG gene rearrangements in these cases was most likely due to unusual V(D)J rearrangements or mutations affecting primer binding sites.

Clonal IG gene rearrangements were detected in five of 24 (21%) staging or follow-up BM aspirates in which there was no morphological or immunophenotypic evidence of disease. These were staging samples for DLBCL (3) and MALT lymphoma (1) and follow-up for myeloma (1). The clonal IG gene rearrangements detected in these cases were probably derived from small numbers of morphologically undetectable clonal neoplastic cells associated with the respective primary lymphomas. Clonal IG gene rearrangements detected in other LPD are detailed in Table I.

Relative detection rate by individual and combined BIOMED-2 reactions. The rates of detection of clonal IG gene rearrangements by the BIOMED-2 reactions, used singly and in combination, were calculated in 159 specimens (54 fresh/frozen and 105 paraffin) that had adequate DNA quality and showed unequivocal clonal results. When analysed separately in fresh/frozen and paraffin specimens, the rates by reactions IGH\textsubscript{A,D} were lower in paraffin specimens (33–51%) than in fresh/frozen specimens (48–76%) whereas those by other reactions including IGK\textsubscript{A} and IGK\textsubscript{B} were similar between the two specimen types. However, direct comparisons between fresh/frozen and paraffin specimens for each of the reactions were not possible as tissues with both specimen types were not available. In addition, the spectrum of conditions for the two specimen types was different. There were more germinal centre (GC) and post-GC B-cell malignancies within the paraffin group (94/97, 97%, including 29 FL) than in the fresh/frozen group (27/38, 71%, including 3 FL), which had most likely contributed to the differences in detection rates by reactions IGH\textsubscript{A,D}. As shown below, GC/post-GC B-cell neoplasms, particularly FL, showed much lower clonality detection rates with IGH\textsubscript{A,D} than did pre-GC B-cell malignancies. When FL

Table I. Detection of clonal IG gene rearrangements in specimens with suspected B-cell lymphoproliferative disorders (LPD).

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. of clonal specimens/</th>
<th>No. of specimens tested (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diagnostic specimens with no evidence of B-cell neoplasms</td>
<td>0/70 (0)</td>
<td></td>
</tr>
<tr>
<td>Mature B-cell neoplasms</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MALT lymphoma</td>
<td>31/32 (97)</td>
<td></td>
</tr>
<tr>
<td>Follicular lymphoma</td>
<td>30/32 (94)</td>
<td></td>
</tr>
<tr>
<td>Diffuse large B-cell lymphoma</td>
<td>25/26 (96)</td>
<td></td>
</tr>
<tr>
<td>Plasma cell neoplasms</td>
<td>19/20 (95)</td>
<td></td>
</tr>
<tr>
<td>Small lymphocytic lymphoma/ chronic lymphocytic leukaemia</td>
<td>10/10 (100)</td>
<td></td>
</tr>
<tr>
<td>Primary cutaneous follicle centre lymphoma</td>
<td>5/5 (100)</td>
<td></td>
</tr>
<tr>
<td>Mantle cell lymphoma</td>
<td>4/4 (100)</td>
<td></td>
</tr>
<tr>
<td>Burkitt lymphoma</td>
<td>3/3 (100)</td>
<td></td>
</tr>
<tr>
<td>Splenic marginal zone lymphoma</td>
<td>2/2 (100)</td>
<td></td>
</tr>
<tr>
<td>Primary effusion lymphoma</td>
<td>1/1 (100)</td>
<td></td>
</tr>
<tr>
<td>B-cell non-Hodgkin lymphoma unclassified</td>
<td>17/18 (94)</td>
<td></td>
</tr>
<tr>
<td>Subtotal</td>
<td>147/153 (96)</td>
<td></td>
</tr>
<tr>
<td>Staging/follow-up bone marrows with no morphological/phenotypic evidence of B-cell LPD</td>
<td>5/24 (20)</td>
<td></td>
</tr>
<tr>
<td>Other B-cell LPD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post-transplant LPD, polymorphic</td>
<td>4/6 (67)</td>
<td></td>
</tr>
<tr>
<td>Post-transplant LPD, monomorphic</td>
<td>4/4 (100)</td>
<td></td>
</tr>
<tr>
<td>Castleman's disease</td>
<td>2/3 (67)</td>
<td></td>
</tr>
<tr>
<td>Classical Hodgkin lymphoma</td>
<td>1/1 (9)</td>
<td></td>
</tr>
<tr>
<td>T-cell LPD</td>
<td>1*/10 (10)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>164/281 (58)</td>
<td></td>
</tr>
</tbody>
</table>

*Ap a paraffin specimen of transformed mycosis fungoides with EBV positive B-cells.
was excluded from the analysis, the differences in detection rates for IGHA, between the paraffin and fresh/frozen groups were reduced (IGHA-B, IGHB) or eliminated (IGHA-C). The detection rates for fresh/frozen and paraffin specimens were therefore combined for the analyses detailed in Table II.

The mean detection rates for clonal IG gene rearrangements by reactions IGHA, and IGKA, ranged from 50% to 58% whilst those for IGH, IGL and IGHE were much lower. In 31 cases (20%) clonality was demonstrated by only one reaction. The sole use of any individual reaction is therefore of limited value. To identify the combinations of reactions that are capable of achieving the highest detection rates with the least number of reactions, stepwise combinations of two or more reactions were constructed systematically using the six most sensitive reactions (IGHA, and IGKA,). The results of all combinations are summarised in Table II. The most sensitive two-reaction combinations were those consisting either of IGHA or IGHB together with either of IGKA or IGKB, achieving detection rates from 77% to 81%. The most sensitive three-reaction combination was ‘IGHB + IGKA+B’, achieving a detection rate of 91%, of which 58% were detected by at least two reactions. Addition of IGHA and/or IGHD increased the detection rate to 99%, of which 79% were detected by at least two reactions. These combinations, which included reactions for both IG heavy and light chains, were superior to those analysing IG heavy or light chains alone (all \(P < 0.05\)).

The rate of detection of clonality was further determined in 126 clonal specimens with pre-GC (13) or GC/post-GC (113) B-NHL (Table III). The clonality detection rate of individual reactions was consistently higher in the pre-GC group than in the GC/post-GC group. This was most significant for the three reactions IGHA, as each of them showed a 100% detection rate in pre-GC lymphomas but a detection rate of no greater than 52% in the GC/post-GC group and 30% in FL. Using the same approach as described above, ‘IGHB + IGKA+B’ was again
Table III. Rate of clonal IG gene rearrangements detected by individual and combined BIOMED-2 reactions in different subtypes of mature B-cell malignancies.

<table>
<thead>
<tr>
<th>PCR reactions</th>
<th>Pre-germinal centre (%)</th>
<th>Germinal centre and post-germinal centre (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MCL (n = 4)</td>
<td>SLL/CLL (n = 9)</td>
</tr>
<tr>
<td>IGH_A</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>IGH_B</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>IGH_C</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>IGH_D</td>
<td>75</td>
<td>67</td>
</tr>
<tr>
<td>IGH_E</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>IGH_A</td>
<td>75</td>
<td>100</td>
</tr>
<tr>
<td>IGH_B</td>
<td>50</td>
<td>67</td>
</tr>
<tr>
<td>IGL</td>
<td>75</td>
<td>44</td>
</tr>
</tbody>
</table>

Best combinations of two or more reactions

- IGH_A + IGK_A
- IGH_A + IGK_B
- IGH_B + IGK_A
- IGH_B + IGK_B
- IGH_A + IGH_B + IGK_B
- IGH_B + IGH_D + IGK_B
- IGH_A + IGK_A + IGK_B
- IGH_B + IGH_D + IGK_A + IGK_B
- IGH_A + IGK_B
- IGH_A + IGK_C
- IGK_A + IGK_B

Combinations of IG heavy/light chain reactions alone

- IGH_A + IGH_B + IGH_C
- IGH_A + IGH_B + IGH_C + IGH_D + IGH_E
- IGK_A + IGK_B
- IGK_A + IGK_B + IGL

For cases with identical clonal rearrangements in multiple specimens, only one specimen was included for each case.

- MCL, mantle cell lymphoma; SLL/CLL, small lymphocytic lymphoma/chronic lymphocytic leukaemia; FL, follicular lymphoma; MALTL, MALT lymphoma; DLBCL, diffuse large B-cell lymphoma; PCN, plasma cell neoplasms; Others: five primary cutaneous follicle centre lymphoma, three Burkitt lymphoma, two splenic marginal zone lymphoma, one primary effusion lymphoma.

identified as the best three-reaction combination for GC/post-GC lymphomas with a mean detection rate of 91%. Specifically for FL, the detection rate was 97% using ‘IGH_B + IGK_A+B’, but only 37% using the three reactions IGH_A+B+C and 57% using all five IGH reactions (all P < 0.01).

Detection of clonal TCR gene rearrangements in mature T-cell LPD

Sensitivity and specificity. Using the full set of BIOMED-2 reactions for T-cell clonality, clonal TCR gene rearrangements were detected in 56 of 57 (98%) specimens diagnosed with mature T-cell neoplasms (21 of 21 fresh/frozen (100%) and 35 of 36 paraffin (97%) specimens). Failure to detect a clonal TCR gene rearrangement in one specimen diagnosed with an unspecified peripheral T-cell lymphoma was most probably due to unusual V(D)J rearrangements at the TCR loci. Polyclonal or no clonal TCR gene rearrangements were obtained in 38 of 41 (93%) diagnostic specimens without evidence of T-cell malignancy (13 of 15 fresh/frozen (87%) and 25 of 26 paraffin (96%) specimens) (Table IV). These results indicated that the BIOMED-2 PCR assays for TCR gene rearrangements were clinically highly sensitive and specific. There was no difference between fresh/frozen and paraffin specimens in these respects (all P > 0.05).

Three diagnostic specimens showed no morphological or immunophenotypic evidence of a T-cell malignancy but clonal TCR results. One of these was a BM aspirate showing red cell aplasia in which the clonal result might be attributed to the presence of an occult clonal population of large granular T lymphocytes (Go et al, 2000); one was a BM aspirate in a patient previously showing CD8+ lymphocytosis; one was a BM aspirate showing red cell aplasia in one patient previously showing CD8+ lymphocytosis; one was a BM aspirate showing red cell aplasia in which the clonal result was also associated with inflammation (Hingorani et al, 1993).
lymphoma in which the clonal result may have been derived from a small number of morphologically and immunophenotypically undetectable neoplastic T cells. The other one was a follow-up sample for B-cell post-transplant LPD and the clonal result in this case might reflect clonal or oligoclonal T-cell expansion associated with immunodeficiency (Wang et al., 2000). The clonal TCR gene rearrangements detected in other diseases are detailed in Table IV.

Relative detection rate by individual and combined BIOMED-2 reactions. The rates of detection of clonal TCR gene rearrangements by the BIOMED-2 reactions, used singly and in combination, were evaluated in 66 specimens (26 fresh/frozen and 40 paraffin) that showed unequivocal clonal results associated with immunodeficiency (Wang et al., 2000). The clonal TCR gene rearrangements detected in other diseases are detailed in Table IV.

Relative detection rate by individual and combined BIOMED-2 reactions. The rates of detection of clonal TCR gene rearrangements by the BIOMED-2 reactions, used singly and in combination, were evaluated in 66 specimens (26 fresh/frozen and 40 paraffin) that showed unequivocal clonal results by at least one reaction. Detection rates were first calculated separately for fresh/frozen and paraffin specimens. As detection rates for each of the six individual reactions were very similar between the two types of specimens (data not shown), the data were combined (Table V, Table SIII). TCRGA was the most sensitive single reaction, showing a detection rate of 80%. TCRGβ and each of three TCRB reactions showed detection rates of around 40%. TCRD reaction showed a detection rate of 28%. Eleven specimens (17%) demonstrated clonality in one reaction only. TCRGA and TCRGβ, two complementary reactions for TCRG gene rearrangements, gave a combined clonality detection rate of 94%. TCRBα and TCRBβ, which are complementary and target complete TCRB gene rearrangements, showed a combined detection rate of 66% and the addition of TCRBγ for incomplete TCRB gene rearrangement increased the detection rate to 76%. When combinations of the six reactions for TCR gene rearrangements were assessed using the same strategy as for IG gene rearrangement analysis, the most sensitive two-reaction combination was the TCRGA and TCRGβ combination. The addition of TCRBα increased the detection rate to 98%. The further addition of TCRBβ and TCRBγ, although not increasing the rate of detection of clonality, increased the proportion of cases shown to be clonal by at least two reactions to over 70%, thus offering a higher level of confidence in clonality interpretation. When the detection rate was assessed in different categories of mature T-cell malignancies, similar data were observed (Table SIV).

Discussion

A number of studies have reported the successful application of BIOMED-2 assays to the investigation of IG or TCR gene rearrangements in LPD (Matthews et al., 2004, 2006;
Morgan et al., 2006). A few studies have also assessed subsets of BIOMED-2 PCR primers for clonality analysis at diagnosis in selected specimen types (Lassmann et al., 2005; Lukowsky et al., 2006) or disease sub-categories (Sandberg et al., 2003; Droese et al., 2004; Catherwood et al., 2006) and demonstrated improved clonality detection compared with previously published PCR or Southern-blot methods. Since our study was initiated, two studies evaluated BIOMED-2 assays on routine clinical samples and concluded that these assays have high clinical sensitivity and specificity for B-NHL (McClure et al., 2006) or disease sub-categories (Sandberg et al., 2003; Droese et al., 2004; Catherwood et al., 2006) and demonstrated improved clonality detection compared with previously published PCR or Southern-blot methods. Since our study was initiated, two studies evaluated BIOMED-2 assays on routine clinical samples and concluded that these assays have high clinical sensitivity and specificity for B-NHL (McClure et al., 2006) and can reliably replace Southern-blot analysis in routine clonality analysis (Sandberg et al., 2005). More recently, the participants of the BIOMED-2 collaborative group reported further studies using these PCR assays in large series of B- and T-cell malignancies and reactive lesions and validated their utility in the reliable detection of clonality in lymphoid malignancies (Bruggemann et al., 2007; Evans et al., 2007; Langerak et al., 2007). In addition, a guideline for use of these assays in routine clonality testing was proposed (van Krieken et al., 2007). However, these studies used only selected fresh or frozen samples with a considerable yield of high quality DNA. The other studies referred to above also focused primarily on fresh materials. Where paraffin specimens were included, they were tested with only some of the BIOMED-2 primer sets for IG genes in either a small number of cases (McClure et al., 2006) or using specimens from selected tissue sites (Lassmann et al., 2005; Lukowsky et al., 2006). Other than the initial BIOMED-2 study (in which very few paraffin specimens were tested), no studies have assessed the BIOMED-2 primers for detection of TCR gene rearrangements in paraffin-embedded tissues. We believe that a comprehensive, independent evaluation of the BIOMED-2 assays in routine paraffin specimens is essential as paraffin-embedded tissues constitute the majority of specimens in routine haematopathological diagnosis. Furthermore, the quality of DNA extracted varies considerably due to the effect of tissue fixation and processing and many biopsies are small and inadequate for standard DNA purification procedures as suggested by the BIOMED-2 studies.

The present study is the first to prospectively assess the use of BIOMED-2 PCR assays for IG and TCR gene rearrangements in routine haematopathological practice based largely upon paraffin specimens. Among a total of 441 specimens analysed, 316 had valid PCR results from all six reactions in each specimen. Two specimens had no results from TCRB reactions and one specimen had no result from TCRD reaction. The complete sets of the combinations are listed in Table SIII.

### Table V. Rate of clonal TCR gene rearrangements detected by individual and combined BIOMED-2 PCR reactions in all specimens with clonal TCR gene rearrangements.

<table>
<thead>
<tr>
<th>PCR reactions</th>
<th>No. of cases clonal by at least one reaction/ no. of cases tested* (%)</th>
<th>No. of cases clonal by two or more reactions (%)</th>
<th>No. of cases clonal by indicated reaction only (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCRBA</td>
<td>29/64 (45)</td>
<td>2 (3)</td>
<td></td>
</tr>
<tr>
<td>TCRBB</td>
<td>24/64 (38)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>TCRBC</td>
<td>24/63 (38)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>TCRGA</td>
<td>53/66 (80)</td>
<td>2 (3)</td>
<td></td>
</tr>
<tr>
<td>TCRGB</td>
<td>29/66 (44)</td>
<td>6 (9)</td>
<td></td>
</tr>
<tr>
<td>TCRDC</td>
<td>19/65 (28)</td>
<td>1 (2)</td>
<td></td>
</tr>
</tbody>
</table>

Best combinations of two or more reactions

| TCRGA + TCRBB | 52/63 (83)                                      | 23 (37)                                       |
| TCRGA + TCRBC | 54/64 (84)                                      | 22 (34)                                       |
| TCRGA + TCRB  | 57/65 (88)                                      | 14 (22)                                       |
| TCRGA + TCRBC | 56/64 (88)                                      | 25 (39)                                       |
| TCRGA + TCRB  | 62/66 (94)                                      | 20 (30)                                       |
| TCRGA + TCRB  | 60/64 (94)                                      | 35 (58)                                       |
| TCRGA + TCRB  | 62/65 (95)                                      | 31 (50)                                       |
| TCRGA + TCRB  | 60/63 (95)                                      | 34 (57)                                       |
| TCRGA + TCRB  | 63/64 (98)                                      | 38 (60)                                       |
| TCRGA + TCRB  | 63/64 (98)                                      | 46 (73)                                       |
| TCRAG + TCRB  | 62/63 (98)                                      | 47 (76)                                       |
| TCRAG + TCRB  | 63/63 (100)                                     | 42 (67)                                       |
| TCRAG + TCRB  | 62/63 (98)                                      | 49 (79)                                       |

Combinations of reactions for TCRB gene

| TCRBB + TCRB  | 42/64 (66)                                      | 11 (17)                                       |
| TCRBB + TCRB  | 48/63 (76)                                      | 26 (41)                                       |

*For cases with identical clonal rearrangements in multiple specimens, only one specimen was included for each case. Among 66 specimens analysed, 63 had valid PCR results from all six reactions in each specimen. Two specimens had no results from TCRB reactions and one specimen had no result from TCRD reaction. The complete sets of the combinations are listed in Table SIII.
heteroduplex-PAGE analysis, the overall clinical sensitivity and specificity of the combined BIOMED-2 PCR assays were 96% and 100%, respectively, for B-cell clonality and 98% and 93%, respectively, for T-cell clonality. Although a direct comparison between fresh/frozen and paraffin specimens was not possible in our study, there were no apparent differences in the sensitivity and specificity of the combined BIOMED-2 PCR assays for either B- or T-cell clonality analysis. Our results are consistent with a recent study that showed equivalent clinical sensitivity of BIOMED-2 IGH primers on 55 clinical samples with corresponding fresh and paraffin tissues (McClure et al., 2006) and support the original BIOMED-2 report that these assays are applicable to paraffin tissues provided that the DNA samples extracted are adequate for amplification of DNA fragment of greater than 300 bp (van Dongen et al., 2003).

It should be noted that 89% of our paraffin specimens with DNA quality suitable for BIOMED-2 primers were analysed as crude DNA extracts and the majority of them showed amplifiable DNA products of 400 bp or more and produced reproducible clonality results without a need for repetition. Therefore, further purification of crude DNA extracts from routine paraffin specimens seems unnecessary. However, as the DNA concentration in the crude tissue digest could not be quantified, it was important to perform all PCR assays in duplicate with a different amount of DNA template in each sample. If poor amplification or non-reproducible amplification was noted, the amount of DNA template was adjusted and amplification repeated.

Twenty-one per cent of paraffin specimens yielded PCR products less than 300 bp. This percentage, although substantial, is similar to that in a recent investigation of 43 paraffin-embedded skin biopsies using the same protocol (Lukowsky et al., 2006) but markedly lower than the 47% reported in the original BIOMED-2 study (van Dongen et al., 2003). The proportion of specimens with poor DNA quality was variable among tissue types, BM trephine biopsies often being particularly poor. Whereas prolonged fixation and decalcification may have fragmented DNA excessively in these samples, the reason for poor DNA quality in other types of tissue is unclear. The possible presence of inhibitory factors seems unlikely as purification of the remaining crude DNA extract did not improve DNA quality (H. Liu, K. Payne & M.-Q. Du, unpublished results). However, other workers reported improved DNA quality following purification of crude BM lysates using similar methods (Lassmann et al., 2005). It is of note that specimens with amplifiable DNA products less than 300 bp may still be amenable for analysis with those BIOMED-2 reactions that target smaller gene fragments. Indeed, we successfully detected clonal rearrangements in some of these samples using, for example, reactions IGHc and TCRG. Nevertheless, extra caution is required when interpreting clonality results in such specimens.

An important objective of our study was to develop a practical and efficient strategy for the routine use of BIOMED-2 PCR assays as these assays include a relatively large number of overlapping/complementary reactions for both IGH and TCR genes. For this purpose, all specimens with adequate DNA quality were analysed with all the available BIOMED-2 reactions. We found that the utility of each individual reaction when used alone was limited and varied amongst disease subtypes, especially in B-cell malignancies. For example, the mean detection rates for clonal IGH gene rearrangements by reactions IGHcA and IGHcB ranged from 50% to 58% whilst those for IGHcI, IGL and IGHcE were much lower. IGHcA,C each demonstrated clonality in 100% of pre-GC lymphoid malignancies but in no more than 52% of GC-post-GC malignancies and 13–30% of FL, consistent with the presence of frequent somatic hypermutations in the rearranged VDJ gene region leading to inefficient primer annealing (Diss et al., 1993). These results in general agree with those reported by the BIOMED-2 group (Evans et al., 2007), highlighting the need to use BIOMED-2 reactions selectively and, more importantly, in combination for assessment of clonality.

Knowing the detection rate of each individual reaction, we were able to examine all possible combinations of reactions step by step and to identify the best two or more reactions for clonality detection in an objective and logical way. For IGH gene rearrangements, IGKcA and IGKcE were shown to combine with either IGHcA or IGHcI to generate the best two-reaction combinations for B-cell clonality analysis. The three-reaction combination of IGHcB + IGKcA,B achieved a remarkable 91% clonality detection rate both overall and in GC/post-GC lymphoid malignancies, and a 97% detection rate in FL. By contrast, the three reactions targeting the IGH framework regions (IGHcA+B+C) achieved only a 77% detection rate overall, a 71% detection rate in GC/post-GC malignancies and 37% detection rate in FL. The significance of IGK gene loci as the target of clonality analysis has been emphasised previously (Diss et al., 2002; van Dongen et al., 2003; Sandberg et al., 2005; Catherwood et al., 2006; McClure et al., 2006), and been demonstrated further in the newly published study of the BIOMED-2 group, where the combined two IGK reactions showed the same rate of clonality detection as that shown by the combined three IGH Vc1–Jc1 reactions across all five categories of 369 mature B-cell malignancies (Evans et al., 2007). This is consistent with evidence that IGK is rearranged in all IGK positive and virtually all IGL positive B-cells (van der Burg et al., 2001) and, in GC and post-GC malignancies, is either less prone to mutation than the IGH locus (Kosmas et al., 1998) or is unmaturated (IGK-Kde rearrangement) (van Dongen et al., 2003). The addition of one or more of the remaining reactions for IG genes is only needed for a small number of cases or in order to confirm clonality using more than one reaction. The additional value of using the IGL and IGHcE reactions is minimal since each assay was the sole reaction to detect a clonal IGH gene rearrangement in only a single case in our series.

The detection rates also varied markedly for BIOMED-2 TCR gene reactions depending on the target loci. The TCRGJ reaction, which contains the primers targeting the most frequently used VcT1 gene family in VcT-Jc1 rearrangement (van
Dongen et al., 2003), detected clonality in 80% of clonal T-cell LPDs. The combined use of the two TCRG reactions showed a 94% detection rate consistent with the presence of a TCRG gene rearrangement in the majority of mature T-cell malignancies (McCarthy et al., 1992; van Dongen et al., 2003; Hodges et al., 2005; Bruggemann et al., 2007). By contrast, the combined use of three TCRB reactions detected clonality in 85% of mature T-cell malignancies, slightly lower than those reported by van Dongen et al. (2003) and Bruggemann et al. (2007) but similar to other studies (Droese et al., 2004; Sandberg et al., 2005). It should be noted that the cases in our series were unselected and the T-cell malignancies analysed were not assessed for TCRab or TCRcd lineage. Differences in the types of T-cell LPD studied could also have contributed to the differences in the detection rates observed.

In the present study, the most sensitive combinations of three or more reactions were the two TCRG reactions plus one or more of the remaining reactions. From a practical point of view, the two TCRG reactions appeared to be a good choice for starting clonality analysis on routine specimens with a suspected T-cell malignancy, especially on paraffin specimens in which the lineage of the T-cell malignancy often cannot be determined. However, TCRG loci have a restricted germline repertoire and limited junctional diversity at the rearranged Vc-Jc region, and thus theoretically carry the risk of producing pseudoclonality in samples containing small numbers of T-cells (van Dongen et al., 2003). Therefore, although in practice we did not encounter significant problems in the interpretation of results from the TCRG reactions, addition of one or more of the remaining reactions may be useful to confirm clonality. In this case, one or more of the TCRB reactions is preferred over the TCRD reaction. This is because the majority of T-cell malignancies are TCRzβ+, and thus harbour a rearranged TCRB gene, and these combinations are more likely to detect clonal rearrangements by two or more reactions. Moreover, the TCRD has a limited repertoire of VDJ recombination and there is restricted usage of Vβ gene segments with age leading to oligoclonality (van Dongen et al., 2003). However, for the minority of LPDs known or suspected to be of TCRγδ lineage, TCRD can be used, together with TCRBc as incomplete Dβ-Jβ rearrangement is frequently present in this type of tumour (Droese et al., 2004).

Based on the above assessment and with the aim of presenting an efficient approach, we propose a novel, step-wise strategy for the routine application of BIOMED-2 PCR assays which delivers sensitive detection of clonality using the fewest reactions possible for initial testing (Fig 1). The three reactions IGHb + IGKα+β and the two TCRG reactions detected clonality in over 90% of cases with clonal IG and TCR gene rearrangements respectively. These reactions are therefore

![Diagram](image-url)

**Fig 1.** A practical strategy for the routine use of BIOMED-2 PCR assays for B- and T-cell clonality in both fresh and fixed specimens with a suspected lymphoproliferative disorder (LPD). In cases with a suspected B-cell LPD, a single pathway can be followed. In any case with a suspected T-cell LPD, analysis may follow the main pathway indicated. However, if the lineage of the T-cell proliferation is known (TCRαβ versus TCRγδ) the appropriate alternate pathway indicated may be used (The percentage in parenthesis indicates the rate of detection of clonality by at least two reactions).
recommended as the first-line reactions for B-cell and T-cell clonality analysis. Further testing, again in a stepped manner, is only needed in a small number of cases or in order to confirm clonality using additional reactions.

Our strategy is analogous to the approach proposed by the BIOMED-2 group (van Krieken et al., 2007), but has important differences in the choice of first-line reactions, particularly in the analysis of B-cell clonality. van Krieken et al. (2007) recommend the initial use of the three IGH V_{H} J_{H} reactions, followed by (or preferably together with) the two IGK reactions as the first-line reactions for B-cell clonality analysis.

Since both our study and that of BIOMED-2 show that clonality can be detected in around 90% of B-cell lymphomas by the combined use of ≤3 reactions, it is appropriate, particularly in view of cost efficiency, to begin analysis with the three reactions that give the highest sensitivity. We recommend IGH \_B + IGK_{A+B} rather than the three V_{H} J_{H} reactions since our results unequivocally demonstrate that IGH \_B + IGK_{A+B} (91%) is far more sensitive than the three IGH V_{H} J_{H} reactions in combination (77%). This increased sensitivity probably reflects the greater complementarity of IGH and IGK reactions that target independent gene rearrangements, in contrast to the three V_{H} J_{H} reactions that target the same gene rearrangement. In addition, IGH, particularly the CDR3 region, is prone to somatic hypermutation that may reduce the sensitivity of the V_{H} J_{H} reactions (Jolly et al., 1996). The increased detection of clonality by combined analysis of IGK and IGH over that by analysis of each locus alone is also demonstrated in the BIOMED-2 study (Evans et al., 2007).

The BIOMED-2 study recommends the use of the three TCRB reactions and the two TCRG reactions (either sequentially in either order or, preferably, in combination) in the initial assessment of clonality in the majority of mature T-cell lymphoproliferations. Since in our prospective analysis of routine samples, the combination of the two TCRG reactions was slightly more sensitive than the combination of the three TCRB reactions and allowed the detection of clonality in 94% of T-cell malignancies, we suggest that this combination represents a cost efficient initial panel of reactions and that the use of five reactions may not be needed in the vast majority of cases.

In summary, the present study demonstrated that the BIOMED-2 assays are highly efficient for the detection of clonality in routine haematopathological diagnosis with 96% and 97% detection rates for mature B- and T-cell malignancies respectively. The clinical sensitivities and specificities are not significantly different between fresh/frozen and paraffin specimens provided that the DNA extracted can be amplified for PCR products of over 300 bp. We show that the BIOMED-2 assays are best used in selected combinations and propose a stepwise, efficient strategy for the use of BIOMED-2 PCR assays in the routine assessment of suspected mature lymphoid malignancies (Fig 1). This strategy is applicable to both fresh and paraffin tissues and may also be adjusted according to disease type.

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**Supplementary material**

The following supplementary material is available for this article online:

Table S1. Summary of specimens analysed for IG and/or TCR gene rearrangements.

Table SII. Rate of clonal IG gene rearrangements detected by individual and combined BIOMED-2 reactions in all specimens with clonal IG gene rearrangements.

Table SIII. Rate of clonal TCR gene rearrangements detected by individual and combined BIOMED-2 reactions in all specimens with clonal TCR gene rearrangements.

Table SIV. Rate of clonal TCR gene rearrangement detected by individual and combined BIOMED-2 reactions in different subtypes of mature T-cell malignancies.

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