CHAPTER 7  MOLECULAR AND CYTOGENETIC STUDIES  
— DIAGNOSTIC APPLICATIONS

7.1  B-cell clonality testing by PCR for diagnostic purposes

7.1.1  Immunoglobulin gene rearrangements

Assessment of IgH gene rearrangements is the principal approach to B-cell clonality testing. Ig light chain (IgL) gene rearrangement assays are also available but are not routinely used. A number of PCR approaches and detection systems may be used for IgH clonality testing. The most commonly used are complementarity determining region 3 (CDR3) strategies, which amplify the CDR3 region where the greatest junctional diversity is generated during gene rearrangement. Typically, degenerate consensus primers annealing to framework region (FR3) of the variable (V) region genes are used in conjunction with consensus primers to the 3’ ends of the joining (J) region genes in monoplex, hemi-nested or nested assays. Additional reactions using consensus FR1 or FR2 primers, or use of gene family-specific primers (usually directed at FR1 or leader sequences), can increase the frequency of clonal detection.

Qualitative sensitivity of PCR testing for IgH rearrangement

Qualitative sensitivities vary widely, from <50% to virtually 100% of B-cell lymphomas, depending on the assay design, case-mix, primer selection and detection system employed. For example, false negatives are more likely to occur with follicular, marginal zone and diffuse large B-cell lymphomas, owing to V-region somatic hypermutations, particularly in follicular lymphoma, which affects primer annealing in CDR3 assays. Using CDR3 assays with consensus primers (typically FR3 region V primers) and conventional gels, the frequency of clonal detection ranges from approximately 60% to 80%. This increases to >90% of cases by using additional assays employing FR2 and/or FR1 or leader region primers, by including assays for IgL gene rearrangements, by adding assays for specific chromosomal translocations, and by using more sensitive gel systems, including CEGS.

Analytical (quantitative) sensitivity of PCR testing for IgH rearrangement

Using simple CDR3 strategies, consensus primers and non-denaturing gels, analytical sensitivities are in the range of 1–10% clonal cells in a polyclonal background. Greater sensitivity (0.1–1% clonal cells in a polyclonal background) may be achieved with higher resolution denaturing/sequencing gels with or without automated fluorescent DNA fragment analysis.

Specificity and positive predictive value of PCR testing for IgH rearrangement

Specificities of clonal IgH gene rearrangements range from approximately 80% to 100%, and positive predictive values from 70% to 100%.

Interlaboratory variability and standardisation

There is a need for interlaboratory standardisation of assays. Significant interlaboratory variations in qualitative sensitivity have been reported using the same lymphoma samples (range 20–90% frequency of clonal detection), particularly in paraffin-embedded tissue. The BIOMED-2 Concerted Action collaborative study has addressed such deficiencies in PCR clonality testing, and has published standardised primers and protocols for multiplex PCR assays for clonality studies, reporting unprecedentedly high rates of clonal detection.
### Guideline — Interpretation of assay results

<table>
<thead>
<tr>
<th>Level of evidence</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV</td>
<td>2–7</td>
</tr>
</tbody>
</table>

PCR results for IgH clonality testing should:

(i) be interpreted in the context of a detailed knowledge of the nature of the assay used, its qualitative and analytical sensitivities, and predictive value.

(ii) recognise that the most commonly employed CDR3 assays using consensus primers may have a significant false negative rate, particularly in follicular, marginal zone and diffuse large B-cell lymphomas.

### 7.2 T-cell clonality testing by PCR for diagnostic purposes

#### 7.2.1 TCRγ gene rearrangements

Because of its simple genomic structure and the requirement for few Vγ and Jγ primer combinations to detect all possible rearrangements of the gene, the TCRγ gene is the preferred gene for T-cell clonality testing in routine laboratories. As the gene has only four variable region families and five joining segment genes, construction of consensus or gene segment-specific primers is relatively simple. Assays vary in their design and complexity, and a range of different detection systems, including CEGS, may be used, all of which affect the qualitative and analytical sensitivities of the assays.7,10–19

**Qualitative sensitivity**

There is a wide range in the reported frequency of clonal detection (~60% to virtually 100%), reflecting the effects of the case-mix, nature of the PCR assay employed, primer selection and sensitivity of the detection system. By using multiple primer combinations, which will detect all possible TCRγ gene rearrangements, and routine PAGE, clonal detection rates of 80% to 90% may be achieved. This may be increased to >90% and approaching 100% by employing high-resolution complex gels or automated fluorescent DNA fragment analysis.1 Additional testing for TCRβ gene rearrangements (see below), either in separate assays or by including TCRβ primers in multiplex TCRγ and TCRβ primer mixes, will increase the clonal detection rate by as much as 20%.3

**Analytical sensitivity**

Between 1% and 5% of clonal T cells can be detected in a background of polyclonal TCRγ gene rearrangements in non-denaturing polyacrylamide gels, although inferior sensitivities may result using paraffin-embedded tissue.20 A ten-fold increase in sensitivity (0.1–1%) may be achieved with high-resolution complex gels, or by CEGS, which is fast, accurate, has a high analytical sensitivity at least equal to denaturing gradient electrophoresis DGGE (~0.1–1% in a polyclonal T-cell background), and is able to detect >90% of T-NHL (reviewed in Spagnolo et al.1).

**Test specificity and positive predictive value**

As for mature B-NHL, these values range widely and it is difficult to compare data. Both specificities and positive predictive values range from approximately 70% to 100%.1 In cutaneous B-NHL, the incidence of TCRγ and/or TCRβ clonal gene rearrangements may be particularly high21 (i.e. relatively low positive predictive value). Similarly, dual genotypes in mature T-NHL are disproportionately higher in cutaneous cases, compared with non-cutaneous cases.21 In inflammatory skin disorders in particular, PCR assays should be repeated because of the frequent occurrence of pseudoclonal TCRγ rearrangements, which in a single PCR assay could be misinterpreted as being monoclonal.22,23
7.2.2 TCR\(\beta\) gene rearrangements

Because of its complexity, the TCR\(\beta\) gene is used less often in T-cell clonality testing. It has a large germline repertoire that includes numerous V gene families and J segments, thus restricting the design of sensitive but simple assays based on limited numbers of consensus primers. The large intron separating rearranged VDJ segments from C regions largely precludes DNA-based assays using V and C region primers, which requires RT-PCR, adding to the complexity of the assays. A variety of PCR approaches are published, varying in design complexity, qualitative and quantitative sensitivities.\(^1\)

<table>
<thead>
<tr>
<th>Guideline — Interpretation of assay results</th>
<th>Level of evidence</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR analysis of TCR(\gamma) gene rearrangements is the recommended first-line approach for T-cell clonality testing. The results should be interpreted in the context of a detailed knowledge of the qualitative and analytical sensitivities, and the predictive value of the assay used.</td>
<td>IV</td>
<td>7, 10–19</td>
</tr>
</tbody>
</table>

7.3 Minimal residual disease detection and monitoring (MRDDM)

PCR assays, and RQ-PCR in particular, are being used increasingly for MRDDM in lymphoma. The critical interpretation and clinical significance of results requires consideration of technical, biological and clinical factors.\(^24\) There is a need for greater standardisation of methodology and criteria for interpreting results,\(^27\) along the lines proposed by the BIOMED-2 Concerted Action\(^26,27\) and the Europe Against Cancer Program\(^28,29\), but there are significant cost implications in this approach.

7.3.1 Molecular targets in MRDDM

These are essentially the same as those used for primary lymphoma diagnosis, specifically AgR gene rearrangements (which may involve using patient-specific oligonucleotide primers or probes), chromosomal translocations (DNA based) or fusion gene transcripts resulting from chromosomal translocations (RT-PCR). Sensitive assays are needed to achieve the required analytical sensitivities of \(10^{-4}\) to \(10^{-6}\), particularly in AgR gene rearrangement assays where clonal rearrangements need to be distinguished from any background polyclonal rearrangements.\(^1\)

7.3.2 RQ-PCR in MRDDM

RQ-PCR assays are now the preferred approach to MRDDM\(^25\). The choice of strategy depends on the disease category, the nature of the molecular target, the analytical sensitivity required, and the expertise of the laboratory. Each method has potential advantages and disadvantages.\(^25\)

7.3.3 Analytical sensitivity of RQ-PCR, controls and quantitation

Using fusion gene mRNA transcripts as PCR targets, sensitivities of \(10^{-4}\) to \(10^{-6}\) can be achieved, with little risk of false positivity from detection of low-level fusion transcripts present in normal cells. With AgR gene rearrangements as targets, sensitivities between \(10^{-3}\) to \(10^{-5}\) are achieved.\(^35,36\) Control genes must be included in the assays to correct for DNA or RNA/cDNA quality, as this affects product quantitation.\(^25,31\)

7.4 Testing for chromosomal translocations

Recurring chromosomal abnormalities (see Table 7.1) characterise certain non-Hodgkin lymphomas (NHLs) and are used for both diagnostic purposes and MRDDM. They may be detected by a variety of techniques, including SB, DNA-PCR, RT-PCR, classical cytogenetics and FISH. The method of
choice depends on the particular translocation being assessed. Among the most frequently assessed translocations in lymphoma diagnosis are the t(14;18)(q32;q21) in follicular lymphoma, the t(11;14)(q13;q32) of mantle cell lymphoma, and the t(2;5)(p23;q35) of systemic anaplastic large-cell lymphoma (ALCL).

### 7.4.1 t(14;18)(q32;q21)

This is the most common non-random chromosomal translocation occurring in NHL. It is detected by cytogenetics in 80% to 90% of follicular lymphomas and in 20% to 30% of diffuse large B-cell lymphomas. Although genomic PCR may be used for detection, other modalities are more sensitive, namely SB (but PCR is more cost effective), conventional cytogenetics, FISH and fibre-FISH (in increasing order of sensitivity). 32–39

**PCR diagnostic assays for t(14;18)(q32;q21)**

Comparable qualitative sensitivities may be achieved in frozen or paraffin-embedded tissue approaching that of conventional cytogenetics, and optimised assays may achieve analytical sensitivities of 1 in 10^5 cells. 1 Employing two sets of primers specific for both the major breakpoint region and minor cluster region, the translocation will be detected in 60% to 80% of cases. The presence of small numbers of translocation-positive cells in normal individuals and in hyperplastic nodes 1 argue against the use of very sensitive, nested assays, or RQ-PCR assays designed for MRD detection. These potential false positives are avoided by using standard single-primer set diagnostic assays.

**t(14;18) PCR for MRDDM**

Sensitive nested t(14;18) PCR assays with sensitivities of 1 in 10^5–10^6 are used for MRDDM, and for assessing the efficacy of marrow purging prior to autologous transplantation. Where available, RQ-PCR assays are now the preferred method of testing, using TaqMan and LightCycler systems that are at least as sensitive as conventional nested assays. 1 Their high analytical sensitivity mandates caution in interpreting ‘molecular relapse’ in treated patients, as translocation-positive cells in normal individuals can be detected at levels as high as 1 in 10^4 cells. 40

**Interlaboratory variability**

Multi-institutional studies have reported a wide interlaboratory variability in bcl-2 testing methodology, with a large proportion of laboratories not knowing the analytical sensitivity of their system (i.e. the lower limit of detection), and having significant false-positive rates and low sensitivities. 38,41 These indicate the need for greater interlaboratory standardisation for these assays, especially in the setting of MRD detection.

<table>
<thead>
<tr>
<th>Guideline</th>
<th>Level of evidence</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>FISH or PCR assays are the methods of choice for detecting the t(14;18)(q32;q21).</td>
<td>IV</td>
<td>32, 34–38</td>
</tr>
</tbody>
</table>

### 7.4.2 t(11;14)(q13;q32)

This translocation between the CCND1/BCL-1 and IgH genes, which characterises mantle cell lymphoma (MCL) and is rarely found in other lymphomas, results in deregulated cyclin D1 expression. In decreasing order of qualitative sensitivity, the modalities for detecting aberrations of the bcl-1 gene are DNA fibre-FISH (~100%), conventional FISH including interphase FISH (>95%), in situ mRNA hybridisation (>80%), immunohistochemical staining for cyclin D1 protein (range 70% to >90%), conventional cytogenetics (60–70%), SB (~70%), and genomic PCR (most studies <50%). 33,39,42 Almost all translocation-positive MCL will be detected by the various FISH techniques.
available\textsuperscript{36,37,43–46}. Genomic PCR, including real-time quantitative PCR assays, is of limited sensitivity (40–50%), as only translocations involving the major translocation cluster of \textit{bcl-1} will be detected with standard assays. Immunohistochemical demonstration of nuclear cyclin D1 protein expression is the most cost-effective ancillary diagnostic test for MCL, with sensitivities ranging between 70\% and >95\%.\textsuperscript{45–49}

<table>
<thead>
<tr>
<th>Guideline</th>
<th>Level of evidence</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunostaining for cyclin D1 protein is the recommended modality for confirming a diagnosis of mantle cell lymphoma.</td>
<td>IV</td>
<td>45–49</td>
</tr>
<tr>
<td>FISH techniques, if available, are the most sensitive means of demonstrating the t(11;14)(q13;q32).</td>
<td>IV</td>
<td>36, 37, 43–46</td>
</tr>
</tbody>
</table>

### 7.4.3 \(t(2;5)(p23;q35)\)

This translocation between the novel ALK and the NPM genes, which characterises most ALCL of T/null cell phenotype, generates a fusion gene — ALK/NPM — resulting in dysregulated ALK protein expression in nucleus and cytoplasm. At least 20\% of ALCL harbour variant ALK translocations involving a translocation partner other than NPM, but still resulting in dysregulated ALK protein expression restricted to the cytoplasm and/or cell membrane.\textsuperscript{1}

The \(t(2;5)(p23;q35)\) translocation may be detected by a variety of methods. The most sensitive and practical is ALK protein immunostaining\textsuperscript{50,51}, which correlates well with other detection methods.\textsuperscript{52} Virtually all translocation-positive cases can be detected by RT-PCR, but variant 2p23 anomalies will not be detected, false positives from contamination will be missed owing to the constant size of the PCR product, and low-level transcripts present in normal individuals may be over interpreted. Long-range genomic DNA-PCR is the preferred PCR modality to avoid these potential pitfalls, but requires high-quality undegraded DNA.\textsuperscript{1} FISH, including interphase FISH, can also detect the \(t(2;5)(p23;q35)\) and variant 2p23 anomalies.\textsuperscript{1}

<table>
<thead>
<tr>
<th>Guideline — Immunostaining — anaplastic large-cell lymphoma</th>
<th>Level of evidence</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunostaining for ALK protein expression is the recommended test for detecting ALK and anaplastic large-cell lymphoma of T/null cell type</td>
<td>IV</td>
<td>50–52</td>
</tr>
</tbody>
</table>
Table 7.1 Common chromosome translocations in non-Hodgkin lymphomas

<table>
<thead>
<tr>
<th>Chromosome aberration</th>
<th>Lymphoma</th>
<th>Genes involved</th>
</tr>
</thead>
<tbody>
<tr>
<td>t(14;18)(q32;q21)</td>
<td>Follicular</td>
<td>BCL-2, IgH</td>
</tr>
<tr>
<td></td>
<td>Diffuse large B-cell</td>
<td></td>
</tr>
<tr>
<td>t(8;14)(q24;q32)</td>
<td>Burkitt lymphoma</td>
<td>C-MYC, IgH</td>
</tr>
<tr>
<td>t(8;22)(q24;q11)</td>
<td>Burkitt lymphoma</td>
<td>C-MYC, IgL</td>
</tr>
<tr>
<td>t(2;8)(p11;q24)</td>
<td>Burkitt lymphoma</td>
<td>C-MYC, IgK</td>
</tr>
<tr>
<td>t(11;14)(q13;q32)</td>
<td>Mantle cell</td>
<td>CCND1 (cyclin D1; BCL-1), IgH</td>
</tr>
<tr>
<td></td>
<td>B-CLL, small subset</td>
<td></td>
</tr>
<tr>
<td>t(11;18)(q21;q21)</td>
<td>Marginal zone/extranodal MALT</td>
<td>API2, MALT1</td>
</tr>
<tr>
<td>t(14;18)(q32;q21)</td>
<td>Marginal zone/extranodal MALT</td>
<td>MALT, IgH</td>
</tr>
<tr>
<td>t(1;14)(p22;q21)</td>
<td>Marginal zone/extranodal MALT</td>
<td>BCL-10, IgH</td>
</tr>
<tr>
<td>t(1;2)(p22;p12)</td>
<td>Marginal zone/extranodal MALT</td>
<td>BCL-10, IgK</td>
</tr>
<tr>
<td>t(2;18)(p11;q21)</td>
<td>CLL/SLL (5%)</td>
<td>BCL-2, Igκ</td>
</tr>
<tr>
<td>t(18;22)(q21;q11)</td>
<td>CLL/SLL (5%)</td>
<td>BCL-2, Igλ</td>
</tr>
<tr>
<td>t(14;19)(q32;q13)</td>
<td>CLL/SLL (&lt;5%)</td>
<td>BCL-3, IgH</td>
</tr>
<tr>
<td>t(9;14)(p13;q32)</td>
<td>lymphoplasmacytoid lymphoma</td>
<td>PAX5, IgH</td>
</tr>
<tr>
<td>t(3;14)(q27;q32)*</td>
<td>de novo diffuse large B-cell</td>
<td>BCL-6, IgH</td>
</tr>
<tr>
<td>t(3;22)(q27;q11)</td>
<td>de novo diffuse large B-cell</td>
<td>BCL-6, Igλ</td>
</tr>
<tr>
<td>t(2;3)(p12;q27)</td>
<td>de novo diffuse large B-cell</td>
<td>BCL-6, Igκ</td>
</tr>
<tr>
<td>2p13–15 amplification</td>
<td>diffuse large B-cell, extranodal</td>
<td>REL amplification (NFKB family member)</td>
</tr>
<tr>
<td>t(2;5)(p23;q35)**</td>
<td>Anaplastic large cell, T/null</td>
<td>ALK, NPM</td>
</tr>
</tbody>
</table>

* many other bcl-6 translocation partners are also described
** >20% of ALCL harbour variant 2p23 rearrangements involving genes other than NPM as a translocation partner (e.g. TPM3, TFG, ATIC, MSN, CLTCL).1

7.5 Virus detection by in situ hybridisation

A number of viruses are implicated in the development of human lymphomas. EBV is the best known. EBV genomic material may be detected in almost 50% of classical Hodgkin disease, in nearly all endemic Burkitt lymphomas, in nasal-type natural killer/T cell lymphoma, angiocentric B-cell lymphoma (lymphomatoid granulomatosis), post-transplant lymphoproliferative disorders, AIDS-associated lymphomas, and primary effusion lymphomas.55 The presence of EBV can be demonstrated in a number of ways, including SB and PCR, but the method of choice is by EBV
EBER *in situ* hybridisation\(^{54-57}\), which is easily applied in paraffin sections with high sensitivity, and is not expensive.

### 7.6 Standardisation of molecular tests

There is a relative lack of interlaboratory standardisation in molecular testing\(^{58}\), which complicates the comparison of data. Few multicentre studies have addressed this issue. Significant interlaboratory variations in assay methodology and clonality detection rates have been found in TCR gene testing\(^{20}\), IgH gene testing\(^{8}\) and (t(14;18)) detection\(^{38,41}\).

Recent multicentre European collaborative studies have been instituted to optimise and standardise PCR assays for the purposes of clonality studies in lymphoma (BIOMED-2 Concerted Action)\(^{9,59}\), leukaemia diagnosis, and MRD detection (Europe Against Cancer Program)\(^{28,29}\). While this approach to standardisation and improved clonal detection is to be lauded, the complexity and costs involved are major drawbacks in a routine laboratory setting. The need for standardisation and guidelines for assignment of monoclonality becomes even more critical with the increasing use of new and sensitive detection methods, such as CEGS, in order to avoid false positive results.

### 7.7 References


