CHAPTER 6 MOLECULAR AND CYTOGENETIC STUDIES — TECHNIQUES

6.1 Introduction

In most lymphoid proliferations, morphological assessment and immunophenotyping are sufficient to establish a diagnosis. In a minority of difficult cases (5–10%)[1,2], molecular investigation may be required for definitive diagnosis. Approximately 75% of these cases will be resolved by molecular studies.[3,4] Up to 5% of lymphomas defy lineage assessment despite all investigation.[5]

Tissue acquisition and transport (see Section 6.4) are critical in determining the outcome of molecular investigations. To maximise the chances of a meaningful result, communication between the referring laboratory or clinician and the molecular laboratory prior to biopsy or collection of cellular material is important. It is essential to know the limitations, sensitivity and specificity of each test.

Key points

Molecular tests should be performed by laboratories that have the required expertise and participate in relevant quality assurance programs. The results should always be correlated with clinical, morphological, immunophenotypic and other laboratory data, and should never be considered in isolation.

At present, there is no Medicare funding to cover molecular studies. Strategies to overcome this issue should be addressed so that cost does not act as a disincentive.

6.1.1 Indications for molecular testing

- Where not previously determined by morphology and immunophenotyping:
  - demonstration of monoclonality (and presumptive malignancy)
  - determination of lineage
  - determination of the specific lymphoma subtype
- Minimal residual disease (MRD) detection and monitoring (MRDDM)
- Detection of viruses in lymphomas
- Provision of molecular data of possible prognostic relevance

6.1.2 Techniques

i Southern blot (SB) analysis

ii Polymerase chain reaction (PCR), and real-time quantitative PCR (RQ-PCR)

iii Conventional cytogenetics

iv Fluorescence *in situ* hybridisation (FISH)

Other technically demanding and expensive investigative modalities include multi-colour FISH (M-FISH) and spectral karyotyping (SKY), comparative genomic hybridisation (CGH), and gene expression profiling by cDNA microarray technology. These are not presently in general diagnostic use.

6.1.3 Molecular targets

i Antigen receptor (AgR) gene rearrangements:

- Immunoglobulin heavy chain (IgH)
- T-cell receptors (TCRγ and TCRβ)

ii Chromosomal translocations

iii Specific viral sequences

6.2 Techniques

6.2.1 Southern blot (SB) analysis

While PCR-based techniques have replaced SB as the primary molecular diagnostic modality, it still remains the gold standard in clonality testing, and its utility in establishing clonality in diagnostically difficult cases is proven.[6]

Indications for SB

SB is indicated:

- where PCR assays are not possible or are too insensitive
- to detect monoclonal AgR gene rearrangements that are missed in PCR assays.

Advantage of SB analysis

The main advantage of SB analysis is the low false-positive and false-negative rate.[1,7,8]

Disadvantages of SB analysis

- Requires fresh tissue yielding large amounts of high-quality DNA, therefore largely precluding the use of fixed material.
- Time consuming of the order of several days.
- Relatively expensive and labour-intensive procedure.
- Radioactive materials are often used.
- Low analytical sensitivity (see Section 6.2.4 below), thus limiting its utility in entities containing low proportions of monoclonal cells (e.g. T-cell rich B-cell lymphoma; Hodgkin lymphoma) and in MRDDM.

Targets and probes for SB analysis

In routine lymphoma diagnosis, IgH and TCRß gene re-arrangements are analysed. A variety of restriction enzymes, probes and detection systems are available and have been optimised for detecting IgH and TCRß gene rearrangements.[9–12] At least three informative restriction enzymes should be used for each assay to avoid false positives in single digests arising from restriction site polymorphisms or somatic mutations in antigen receptor genes. Apparent clonality in only one of three enzyme digests should be confirmed by using a fourth restriction endonuclease. For IgH and TCRß rearrangements, joining region probes are more informative than constant region probes.[11]

Guideline — Assay — quality assurance	Level of evidence	Refs
Southern blot (SB) protocols should be optimised in each laboratory. At least three informative restriction enzymes should be used for each assay.	IV	9–12

Test performance

SB will detect >90% of B-NHL and T-NHL.[1,11–13] Its analytical sensitivity is 1–5% in a polyclonal background, that is, at least 1–5% clonal cells are needed to be detected as a novel rearrangement.[11,12]

Interpretation of results and pitfalls

Interpretation of SB data and assignment of clonality should be according to accepted guidelines.[11,12,14] False positives may arise from cross-hybridising bands, incomplete DNA digests, restriction fragment-length polymorphisms, transient clonality in abnormal immune states, pseudoclonality in TCRγ assays (this gene has a limited recombinational repertoire), and lineage infidelity (cross-lineage rearrangements), especially in lymphoblastic lymphomas.[6]

Guideline — Assigning clonality	Level of evidence	Refs
Interpretation of Southern blot (SB) data and assignment of clonality should be according to widely accepted guidelines.	IV	11, 12, 14

6.2.2 Polymerase chain reaction (PCR) techniques

Preferred approach to molecular diagnosis

PCR-based assays are now the preferred first-line approach to the molecular diagnosis of lymphomas.[3,15–19] PCR has the following distinct advantages over SB analysis:

- rapid turn-around time
- minimal tissue requirements
- DNA or RNA may be used as templates
- DNA quality is less critical, thus fixed and archival materials may be used
- superior sensitivity enables MRDDM
- radioactive materials are not required
- assays may be automated and multiplexed.

Guideline — Preferred approach to molecular diagnosis	Level of evidence	Refs
Polymerase chain reaction (PCR)-based assays are the preferred first-line approach to the molecular diagnosis of lymphomas.	IV	3, 15–19

Indications for PCR analysis

- To detect clonal rearrangements of the AgR genes.

- To detect recurring chromosomal translocations, which characterise some lymphomas.
- MRDDM.

Specimens suitable for PCR analysis

Because of the less stringent requirements for large amounts of high-quality DNA, a range of specimens are suitable for PCR studies, including:

- small tissue biopsies, for example, from brain, gut
- fine-needle aspiration biopsies
- decalcified bone marrow biopsies
- bone marrow aspirates
- cells scraped from histological or cytological slides
- cells microdissected from tissue specimens.

Archival paraffin-embedded tissue is suitable for many PCR assays and the sensitivity of clonal detection may approach that achieved in fresh specimens.[6] There is significant variation between laboratories in the sensitivity of clonal detection when evaluating paraffin-embedded material, and there is a need for assays to be standardised.[8]

RT-PCR requires good-quality mRNA obtained from fresh specimens of blood, bone marrow or tissue (fresh or immediately snap-frozen). Paraffin wax material is usually unsatisfactory because of RNA degradation.

PCR methodology

PCR assays are either qualitative (most diagnostic assays) or quantitative. Qualitative assays simply detect the presence or absence of a specific genetic event (e.g. AgR gene rearrangement; chromosomal translocation), whereas quantitative assays quantify the PCR product in the setting of MRDDM. There is a wide range of PCR assays available, of varying complexity, cost and sensitivity, and whose designs vary according to the nature of molecular target and whether the assay is for primary diagnosis or for MRDDM.

Many factors affect each assay's sensitivity. They include primer design (whether consensus, gene family-specific or patient-specific), assay design (single primer pair or hemi-nested/nested assays) and PCR product detection systems. There are many gel electrophoretic systems of varying complexity that allow discrimination of PCR products based on their size, or nucleotide sequence and conformation, or DNA melting characteristics, which affects DNA mobility and hence resolution in various types of gels.[6] In routine diagnostic laboratories, non-denaturing polyacrylamide gel electrophoresis (PAGE) is the method most frequently used, combined with either ethidium bromide staining and UV illumination, or hybridisation with labelled probes to visualise the products. Capillary electrophoresis with automated fluorescent DNA fragment analysis (GeneScan) (CEGS) is rapidly becoming a detection system of choice, particularly in academic and research centres, because of its sensitivity and high throughput (see Spagnolo et al.[6] for details). CEGS offers distinct advantages over PAGE and more complex gel systems, but it has some limitations, including significantly higher costs. Its high sensitivity to the level of one base pair increases the potential for misinterpreting pseudoclonality as monoclonality, and strict criteria for interpreting results need to be defined.[6]

PCR assays should be optimised in individual laboratories and carried out according to accepted guidelines for the performance and interpretation of PCR tests.[14] Further, the sensitivity and

limitations of each assay being performed should be known[20,21], bearing in mind that these will need to be reappraised in the setting of new high-resolution automated assays.[22–26]

Guidelines — Assays — quality assurance	Level of evidence	Refs
PCR assays should be optimised in each laboratory, using accepted guidelines for performance and interpretation of results, and with knowledge of the sensitivity and limitations of each assay.	IV	14, 20, 21
In particular, new high-resolution automated assays, including multiplexed assays using comprehensive primer sets, will require a reappraisal of test sensitivities and specificities.	IV	22–26

Test sensitivity of PCR

Two related but different measures of test performance need to be considered when interpreting clonality tests. *Qualitative sensitivity* in the diagnostic setting refers to the percentage of positive (i.e. monoclonal) cases that are detected in a cohort of cases, with reference to a 'gold-standard' benchmark of clonality (e.g. SB). *Analytical sensitivity* is a quantitative measure of the lowest number of clonal cells that need to be present in a sample to be detected by the assay employed. This is affected by a number of biological and methodological factors, including the type of sample, the nature of the cellular background in which the clonal cells are present, and the sensitivity of the detection system, which is critical (e.g. simple gel electrophoresis versus capillary electrophoresis), particularly in MRD testing. As a general guide, for AgR gene rearrangements, using consensus primers and routine gel electrophoresis may achieve, at best, analytical sensitivities of ~1% (one clonal cell in 100 cells), but with a range of ~1–10%, depending on the number of polyclonal cells in the sample. This approach is not sufficiently sensitive for MRD testing. For chromosomal translocations, using either DNA-PCR or RT-PCR may achieve sensitivities of 1 in 10^4–10^6 cells, depending on the nature of the sample, the assay design and the sensitivity of the detection system.

Test specificity of PCR

Cross-lineage rearrangements may occur in lymphomas, particularly in lymphoblastic lymphoma. With some exceptions[3,5], the lack of sufficient detail in published data and the lack of test standardisation make it difficult to draw meaningful conclusions about test specificities and positive predictive values. With the increasing impetus to standardise assays that involve more numerous primer combinations, and with the use of automated high-resolution analysis of PCR products, the frequency of detecting inappropriate AgR gene rearrangements, even in reactive conditions, is likely to increase.[25,26]

6.2.3 Pitfalls in SB and PCR

In both SB and PCR assays, there is the potential for false positive and false negative results secondary to any number of technical and biological factors, as well as from errors in interpretation of results.

False positive results

These may arise from any of the following factors[6]:

- DNA contamination
- nonspecific products from excess amplification cycles, primer-dimer formation or nonspecific primer binding to unrelated DNA sequences through poor primer design
- canonical TCRγ gene rearrangements

- pseudoclonal AgR gene rearrangements as a result of low quantities of target DNA[20,27,28]
- inappropriate AgR gene rearrangements resulting from lineage infidelity, incomplete rearrangements or biclonality
- detection of chromosomal translocations in normal individuals
- the occurrence of clonal lymphoid populations in a range of benign conditions or in the setting of immune dysregulation.

If there is doubt over the assignment of monoclonality versus pseudoclonality in the setting of low quantities of target DNA, PCR assays should be repeated using further DNA from the sample, to ensure that a clone is reproducible.[3,29–31]

Guideline — Assays — quality assurance	Level of evidence	Refs
PCR assays should be performed using a range of target DNA concentrations to avoid misinterpreting as monoclonal any discrete oligoclonal bands resulting from selective amplification of oligoclones in samples containing small numbers of lymphocytes.	IV	20, 27, 28
Where there is doubt over assignment of monoclonality, PCR assays should be repeated to ensure that a clone is reproducible.	IV	3, 29–31

False negative results

These may arise because of:

- sampling errors
- DNA/RNA degradation
- design of the PCR assay, for example, consensus primers will not detect all possible rearrangements of the AgR genes; incomplete AgR rearrangements can be missed if the primer design is not appropriate to detect these
- biological factors, for example, primer mismatch in CDR3 assays as a result of ongoing somatic mutations in the IgH variable region genes, seen particularly in follicular and marginal zone lymphomas.

6.2.4 Cytogenetics

Introduction

Over the past two decades, a large number of chromosomal and genetic abnormalities have been detected in lymphomas. The most prominent are translocations affecting the immunoglobulin heavy chain (IgH) locus on chromosome 14q32. Early investigations were based on conventional karyotype analysis, but in more recent years, developments in molecular cytogenetics ranging from metaphase and interphase fluorescence *in situ* hybridisation (FISH) to multi-colour SKY and comparative genomic hybridisation (CGH) have vastly increased the scope of detection of cytogenetic abnormalities.

It should be noted that whereas interphase FISH can be performed on fixed paraffin-embedded material, other techniques (conventional cytogenetics, metaphase FISH, and SKY) require rapid transport of fresh, viable cells to the laboratory for short-term culture and metaphase production.

Conventional cytogenetics

Since the first cytogenetic abnormality was detected in chronic myeloid leukemia in 1960, chromosome studies have been used to understand the genetic basis of tumorigenesis. Most cytogenetic studies of lymphoma are based on analyses of lymph node specimens. For optimal results, a piece of lymph node should be transported in sterile tissue culture media (RPMI 1640) to the cytogenetics laboratory as soon as possible after excision. A single cell suspension may be obtained by mechanical disaggregation using a scalpel and needle. Short-term culture in RMPI 160 supplemented with 10–20% foetal calf serum has been found by a number of groups to be optimal. Cultures are usually successful only if set up on the same day as the specimen was taken.[32,33]

Chronic B- and T- lymphoid leukaemias are particularly problematic, as they tend to have a low spontaneous mitotic index and a poor response to most common mitogens. The mitogens that have been shown to be most effective in stimulating malignant B cells to divide are TPA and EBV, with TPA the most commonly used. FISH has allowed detection of numerical and structural abnormalities in the majority of cases, overcoming the difficulties inherent in conventional cytogenetic analysis in this group of disorders.

6.2.5 Fluorescence *in situ* hybridisation (FISH)

Introduction

Fluorescence *in situ* hybridisation (FISH) is a valuable technique that allows detection of both structural and numerical chromosomal abnormalities, down to the single cell and single gene level.[34,35]

It has enabled the detection of genetic changes in cases for which conventional cytogenetics has proved uninformative. Virtually any genomic DNA can be used as a probe for interphase and metaphase cells. The most common probes used are those specific for the repetitive sequences at individual chromosome centromeres, whole chromosome paints and locus specific probes.[35] FISH is based on the ability of single-stranded DNA to hybridise to complementary DNA. The target DNA is chromosomal, either in metaphase or interphase cells, and fixed onto a glass slide. The probe is either directly labelled with a fluorescent tag or with a reporter molecule bound to either biotin or digoxigenin. Both the labelled probe and the target DNA are denatured and hybridised together to allow annealing of the complementary sequences. After excess probe is washed away, the directly labelled probes are detected at the site of annealing using fluorescence microscopy. Biotin or digoxigenin labelled probes require the addition of fluorescent-labelled streptavidin or antidigoxigenin antibodies for detection.

Probe types

Centromeric probes: Probes that hybridise to specific chromosome centromeres target the satellite sequences present in the heterochromatin. Centromeric probes are commercially available and allow the number of each chromosome present in either a metaphase or interphase cell to be determined.

Chromosome paints are collections of sequence from the entire length of a specific chromosome, derived from chromosome-specific libraries, flow-sorted chromosomes or microdissected DNA. They allow the identification of complex rearrangements that cannot be determined by conventional cytogenetics and may also reveal cryptic translocations. However, whole chromosome paints are not useful for detecting rearrangements in interphase cells as the un-contracted nature of the chromosome in interphase produces an extremely diffuse signal from which little useful information can be discerned.

Locus-specific probes hybridise to specific sequences and are extremely useful in the identification of translocations. They have been used to identify what are otherwise ‘cryptic’ translocations. Locus-specific probes only provide information on the presence or absence of a particular sequence, and whether particular sequences co-localise. Two locus-specific probes are each labelled with a different coloured fluorescent tag — red and green, for example — and a third colour (yellow) is formed when

the two co-localise, indicating that a translocation has occurred to bring the two loci together. Alternatively, if the two colours co-localise on either side of a particular breakpoint on one chromosome involved in a translocation, the colours separate into their individual colours when a translocation is present.

While FISH has been used most successfully on cytogenetic preparations, a number of methods are now available for extracting cell nuclei from paraffin-embedded sections. Interphase FISH studies have successfully demonstrated translocations in lymphomas using paraffin sections.[36] Interphase FISH may be performed on paraffin-embedded sections in either of the following ways:

- by hybridising directly to thin sections of tissue that have been leeched of paraffin (the advantage of this technique is that it preserves architecture; limitations include overlapping and truncated cells making scoring of individual cells difficult)
- by making individual cell suspensions from thick sections of tissue, to which standard FISH techniques may then be applied.[36,37]

Imprints made on sialinised slides at the time of tissue triage, fixed and stored frozen, are an alternative and inexpensive method of preserving material for possible FISH studies.[38]

Indications for metaphase and interphase FISH

Metaphase and interphase FISH are of proven utility in the detection of numerical chromosomal anomalies (centromeric probes) and translocations (single, dual or triple colour probes) that characterise certain lymphomas.[39–41] Their ability to detect numeric chromosomal abnormalities is a distinct advantage over PCR. They are particularly useful in detecting translocations with widely dispersed breakpoints (e.g. in mantle cell lymphoma and Burkitt lymphoma) that are not readily amenable to PCR analysis.

Advantages and disadvantages of metaphase and interphase FISH

Metaphase FISH has the relative disadvantages of requiring viable cell suspensions. It may be limited in lymphomas with low proliferation rates and where the malignant cells are present only in low numbers. It also has a relatively low resolution of between 2 and 3 Mb, due to the highly condensed nature of DNA in metaphase.

Interphase FISH has the advantage of being applicable to air-dried smears, paraffin sections and nuclei isolated from fresh or frozen tissue, or from paraffin sections. As for metaphase FISH, a variety of probes are suitable for interphase FISH, but unless the probes are of similar stringency they usually cannot be used in combination in a single hybridisation. The resolution of interphase FISH ranges from 100 to1000 Kb (interphase DNA has complex foldings resulting in an inconstant relationship between genomic and physical distances).[41] It is the method of choice to detect translocations where no RT-PCR assays are available, and where breakpoints are widely dispersed. One drawback with interphase FISH in paraffin sections is the overlapping and sectioning of nuclei, which makes it difficult to score individual cells. If available, cytospin and touch preparations are often easier to interpret,.

Advanced techniques

While metaphase and interphase FISH are the most widely used hybridisation techniques in the diagnosis and study of lymphomas, more complex and costly techniques include DNA fibre-FISH, multicolour FISH (M-FISH) and the related SKY, and comparative genomic hybridisation (CGH). The latter techniques require sophisticated digital image capturing and manipulation systems with appropriate software, and are beyond the scope of these guidelines. More detailed discussion and bibliography may be found elsewhere.[6]

6.2.6 Gene expression profiling: cDNA microarray technology

cDNA microarray technology allows for genomic-scale gene expression profiling of lymphomas. While it is mainly a research tool at this point, pathology laboratories play a central role in the harvesting and storage of fresh lymphoma and normal control specimens for these studies, which require undegraded mRNA. For a summary of details relating to methodology and data analysis, refer to Spagnolo et al.[6] Several recent excellent reviews of gene expression profiling in lymphoma are available.[42–46]

Utility of gene expression profiling of lymphoma

Gene expression profiling promises the refinement of lymphoma sub-classification at a molecular level. It may identify genes of potential pathogenetic and predictive significance, and it may direct the development of novel targeted therapies.[46] The clinical utility of such studies has been demonstrated in several lymphoma types. For example, distinct molecular classes of DLBCL have been delineated, which have important outcome differences after anthracycline-based therapy[47,48], while molecular differences have also been shown between early and progressed DLBCL.[49] In multivariate analysis, a model independently predictive of five-year survival after chemotherapy in DLBCL has been achieved.[48] Further, molecular profiling is identifying specific pathogenetic pathways in different molecular groups of DLBCL, with the potential for targeted therapy.[50] Similarly, clinically relevant molecular data are emerging in chronic lymphocytic leukemia, follicular lymphoma and mantle cell lymphoma (see Spagnolo et al.[6] for further details).

6.3 References

1. Spagnolo DV, Taylor J, Carrello S, Saueracker E, Kay PH. Southern blot analysis of lymphoproliferative disorders: use and limitations in routine surgical pathology. Pathology 1994; 26: 268–75.

2. Langerak AW, van Krieken JH, Wolvers-Tettero IL, et al. The role of molecular analysis of immunoglobulin and T cell receptor gene rearrangements in the diagnosis of lymphoproliferative disorders. J Clin Pathol 2001; 54: 565–7.

3. Theriault C, Galoin S, Valmary S, et al. PCR analysis of immunoglobulin heavy chain (IgH) and TcR-gamma chain gene rearrangements in the diagnosis of lymphoproliferative disorders: results of a study of 525 cases. Mod Pathol 2000; 13: 1269–79.

4. Davis RE, Warnke RA, Dorfman RF, Cleary ML. Utility of molecular genetic analysis for the diagnosis of neoplasia in morphologically and immunophenotypically equivocal hematolymphoid lesions. Cancer 1991; 67: 2890–9.

5. Krafft AE, Taubenberger JK, Sheng ZM, et al. Enhanced sensitivity with a novel TCRgamma PCR assay for clonality studies in 569 formalin-fixed, paraffin-embedded (FFPE) cases. Mol Diagn 1999; 4: 119–33.

6. Spagnolo D, Ellis D, Juneja S, et al. The role of molecular studies in lymphoma diagnosis: a review. Pathology 2004; 36: 19–44.

7. van Dongen JJ, Wolvers-Tettero IL. Analysis of immunoglobulin and T cell receptor genes: Part II: possibilities and limitations in the diagnosis and management of lymphoproliferative diseases and related disorders. Clin Chim Acta 1991; 198: 93–174.

8. Bagg A, Braziel RM, Arber DA, Bijwaard KE, Chu AY. Immunoglobulin heavy chain gene analysis in lymphomas: a multi-center study demonstrating the heterogeneity of performance of polymerase chain reaction assays. J Mol Diagn 2002; 4: 81–9.

9. Beishuizen A, Verhoeven MA, Mol EJ, Breit TM, Wolvers-Tettero IL, van Dongen JJ. Detection of immunoglobulin heavy-chain gene rearrangements by Southern blot analysis: recommendations for optimal results. Leukemia 1993; 7: 2045–53.

10. Langerak AW, Wolvers-Tettero IL, van Dongen JJ. Detection of T cell receptor beta (TCRB) gene rearrangement patterns in T cell malignancies by Southern blot analysis. Leukemia 1999; 13: 965–74.

11. Medeiros LJ, Carr J. Overview of the role of molecular methods in the diagnosis of malignant lymphomas. Arch Pathol Lab Med 1999; 123: 1189–207.

12. Cossman J, Fend F, Staudt LM, Raffeld M. Application of molecular genetics to the diagnosis and classification of malignant lymphoma. In: Knowles DM (ed.) Neoplastic Hematopathology. Philadelphia: Lippincott Williams Wilkins, 2001.

13. Griesser H. Gene rearrangements and chromosomal translocations in T cell lymphoma — diagnostic applications and their limits. Virchows Arch 1995; 426: 323–38.

14. O'Leary, TJ, Brindza L, Kant JA, et al. Immunoglobulin and T-cell receptor gene rearrangement assays; approved guidelines. 15, 1–28. 1995.

15. Pan LX, Diss TC, Isaacson PG. The polymerase chain reaction in histopathology. Histopathology 1995; 26: 201–17.

16. Segal GH. Assessment of B-cell clonality by the polymerase chain reaction: a pragmatic overview. Adv Anat Pathol 1996; 195–203.

17. Diss TC, Pan L. Polymerase chain reaction in the assessment of lymphomas. Cancer Surv 1997; 30:21–44.

18. Arber DA. Molecular diagnostic approach to non-Hodgkin's lymphoma. J Mol Diagn 2000; 2: 178–90.

19. Cairns SM, Taylor JM, Gould PR, Spagnolo DV. Comparative evaluation of PCR-based methods for the assessment of T cell clonality in the diagnosis of T cell lymphoma. Pathology 2002; 34: 320–5.

20. Hoeve MA, Krol AD, Philippo K, et al. Limitations of clonality analysis of B cell proliferations using CDR3 polymerase chain reaction. Mol Pathol 2000; 53: 194–200.

21. van der Velden VHJ, Hochhaus A, Cazzaniga G, Szczepanski T, Gabert J, van Dongen JJ. Detection of minimal residual disease in hematologic malignancies by real-time quantitative PCR: principles, approaches, and laboratory aspects. Leukemia 2003; 17: 1013–34.

22. Derksen PW, Langerak AW, Kerkhof E, et al. Comparison of different polymerase chain reaction-based approaches for clonality assessment of immunoglobulin heavy-chain gene rearrangements in B-cell neoplasia. Mod Pathol 1999; 12: 794–805.

23. Beaubier NT, Hart AP, Bartolo C, Willman CL, Viswanatha DS. Comparison of capillary electrophoresis and polyacrylamide gel electrophoresis for the evaluation of T and B cell clonality by polymerase chain reaction. Diagn Mol Pathol 2000; 9: 121–31.

24. Meier VS, Rufle A, Gudat F. Simultaneous evaluation of T- and B-cell clonality, t(11;14) and t(14;18), in a single reaction by a four-color multiplex polymerase chain reaction assay and automated high-resolution fragment analysis: a method for the rapid molecular diagnosis of lymphoproliferative disorders applicable to fresh frozen and formalin-fixed, paraffin-embedded tissues, blood, and bone marrow aspirates. Am J Pathol 2001; 159: 2031–43.

25. Sandberg Y, Heule F, Lam K, et al. Molecular immunoglobulin/T-cell receptor clonality analysis in cutaneous lymphoproliferations. Experience with the BIOMED-2 standardized polymerase chain reaction protocol. Haematologica 2003; 88: 659–70.

26. van Dongen JJ, Langerak AW, Bruggemann M, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia 2003; 17: 2257–317.

27. Taylor JM, Spagnolo DV, Kay PH. B-cell target DNA quantity is a critical factor in the interpretation of B-cell clonality by PCR. Pathology 1997; 309–12.

28. Elenitoba-Johnson KS, Bohling SD, Mitchell RS, Brown MS, Robetorye RS. PCR analysis of the immunoglobulin heavy chain gene in polyclonal processes can yield pseudoclonal bands as an artifact of low B cell number. J Mol Diagn 2000; 2: 92–6.

29. Dippel E, Assaf C, Hummel M, et al. Clonal T-cell receptor gamma-chain gene rearrangement by PCR-based GeneScan analysis in advanced cutaneous T-cell lymphoma: a critical evaluation. J Pathol 1999; 188: 146–54.

30. Assaf C, Hummel M, Dippel E, et al. High detection rate of T-cell receptor beta chain rearrangements in T-cell lymphoproliferations by family specific polymerase chain reaction in combination with the GeneScan technique and DNA sequencing. Blood 2000; 96: 640–6.

31. Dippel E, Klemke D, Hummel M, Stein H, Goerdt S. T-cell clonality of undetermined significance. Blood 2001; 98: 247–8.

32. Juneja S, Lukeis R, Tan L, et al. Cytogenetic analysis of 147 cases of non-Hodgkin's lymphoma: non-random chromosomal abnormalities and histological correlations. Br J Haematol 1990; 76: 231–7.

33. Harrison CJ. The lymphomas and chronic lymphoid leukaemias. In: Rooney DE (ed.) Human cytogenetics: malignancy and acquired abnormalities. Third edn. Oxford: Oxford University Press, 2001.

34. Andreef M, Pinkel D. Introduction to fluorescence in situ hybridization — principles and clinical applications. New York: John Wiley and Sons Inc, 1999.

35. Kearney L. The impact of the new FISH technologies on the cytogenetics of haematological malignancies. Br J Haematol 1999; 104: 648–58.

36. Paternoster SF, Brockman SR, McClure RF, Remstein ED, Kurtin PJ, Dewald GW. A new method to extract nuclei from paraffin-embedded tissue to study lymphomas using interphase fluorescence in situ hybridization. Am J Pathol 2002; 160: 1967–72.

37. Schurter MJ, LeBrun DP, Harrison KJ. Improved technique for fluorescence in situ hybridisation analysis of isolated nuclei from archival, B5 or formalin fixed, paraffin wax embedded tissue. Mol Pathol 2002; 55: 121–4.

38. Katz RL, Caraway NP, Gu J, et al. Detection of chromosome 11q13 breakpoints by interphase fluorescence in situ hybridization. A useful ancillary method for the diagnosis of mantle cell lymphoma. Am J Clin Pathol 2000; 114: 248–57.

39. Vaandrager JW, Schuuring E, Raap T, Philippo K, Kleiverda K, Kluin P. Interphase FISH detection of BCL2 rearrangement in follicular lymphoma using breakpoint-flanking probes. Genes Chromosomes Cancer 2000; 27: 85–94.

40. Frater JL, Tsiftsakis EK, Hsi ED, Pettay J, Tubbs RR. Use of novel t(11;14) and t(14;18) dual-fusion fluorescence in situ hybridization probes in the differential diagnosis of lymphomas of small lymphocytes. Diagn Mol Pathol 2001; 10: 214–22.

41. Kluin PH, Schuuring E. FISH and related techniques in the diagnosis of lymphoma. Cancer Surv 1997; 30:3–20.

42. Staudt LM. Gene expression profiling of lymphoid malignancies. Annu Rev Med 2002; 53:303–18.

43. Lossos IS, Levy R. Diffuse large B-cell lymphoma: insights gained from gene expression profiling. Int J Hematol 2003; 77: 321–9.

44. Schwaenen C, Wessendorf S, Kestler HA, Dohner H, Lichter P, Bentz M. DNA microarray analysis in malignant lymphomas. Ann Hematol 2003; 82: 323–32.

45. Staudt LM. Molecular diagnosis of the hematologic cancers. N Engl J Med 2003; 348: 1777–85 [Erratum in N Engl J Med 2003; 1348(17225): 2588].

46. Wiestner A, Staudt LM. Towards molecular diagnosis and targeted therapy of lymphoid malignancies. Semin Hematol 2003; 40: 296–307.

47. Alizadeh AA, Eisen MB, Davis RE, et al. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature 2000; 403: 503–11.

48. Rosenwald A, Wright G, Chan WC, et al. The use of molecular profiling to predict survival after chemotherapy for diffuse large-B-cell lymphoma. N Engl J Med 2002; 20;346: 1937–47.

49. Nishiu M, Yanagawa R, Nakatsuka S, et al. Microarray analysis of gene-expression profiles in diffuse large B-cell lymphoma: identification of genes related to disease progression. Jpn J Cancer Res 2002; 93: 894–901.

50. Davis RE, Brown KD, Siebenlist U, Staudt LM. Constitutive nuclear factor kappaB activity is required for survival of activated B cell-like diffuse large B cell lymphoma cells. J Exp Med 2001; 194: 1861–74.