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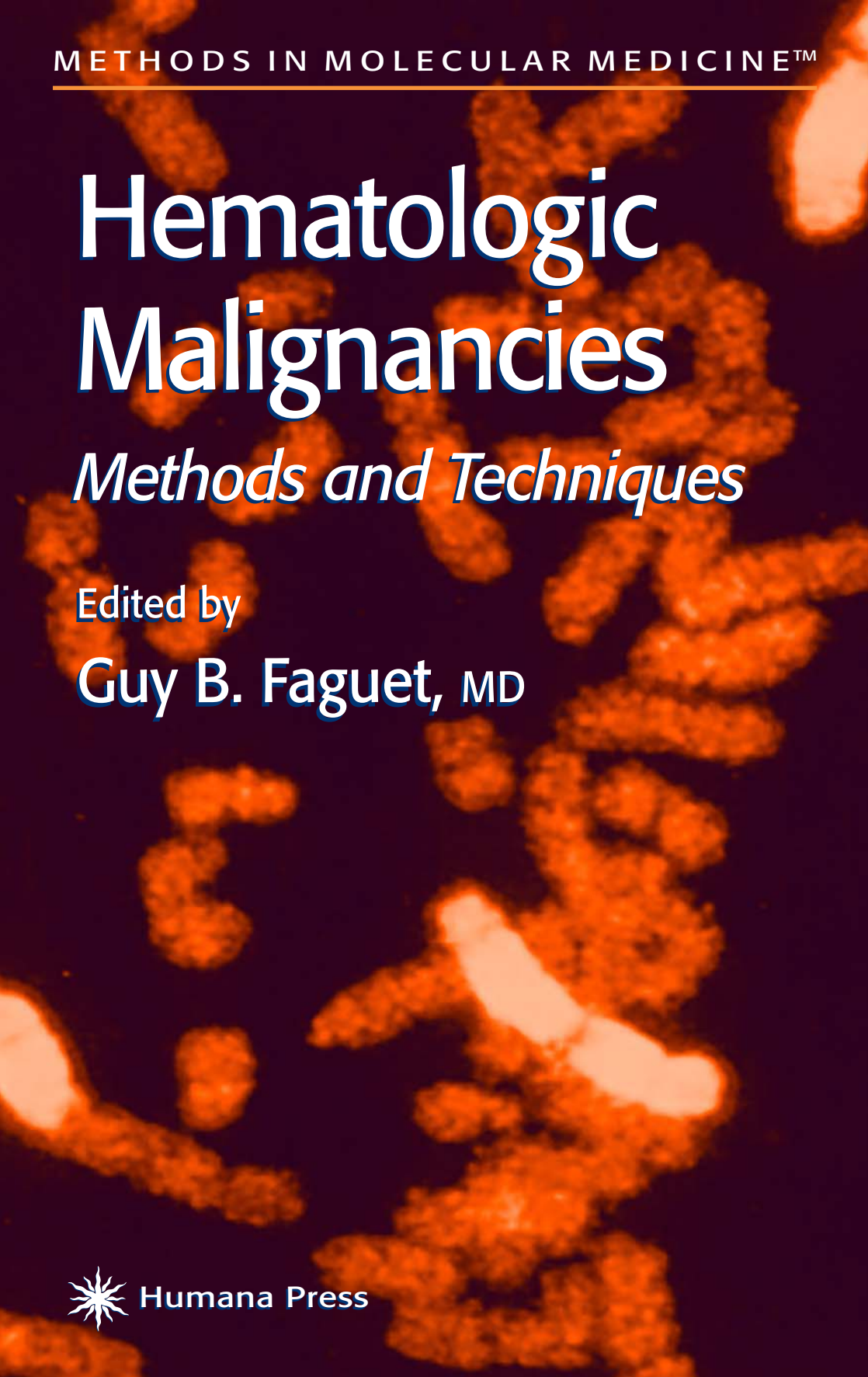
Hematologic Malignancies

Methods and Techniques

Edited by

Guy B. Faguet, MD



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Cover art: FISH using chromosome painting for chromosome 1 in bone marrow metaphase (Fig. 2, page 26).

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Preface

The aim of *Hematologic Malignancies: Methods and Techniques* is to review those methods most useful for the diagnosis and subsequent management of hematologic malignancies. The scope of coverage is intentionally broad, ranging from routine procedures to highly sophisticated methods not currently offered by most clinical laboratories. The latter methods were selected especially to bring into focus recent advances in molecular biology that, since they provide us with strong tools for assessing the outcome of upcoming therapeutic modalities intent on disease eradication, are expected to impact the future diagnosis and management of these diseases. Thus, the common thread among all chapters is clinical relevance, whether sanctioned by past experience or by the expectation that seemingly esoteric research techniques of today will prove clinically valuable in the future. *Hematologic Malignancies: Methods and Techniques* is primarily a compilation of methods presented in sufficient detail—by authors with extensive expertise in their field—to serve not only as a reference for seasoned research and clinical laboratory personnel, but also as a guide for the less experienced. Moreover, the contributing authors also discuss the pathophysiologic bases and the diagnostic usefulness that underscore each method's clinical relevance. Thus, this volume should be also valuable to clinicians—especially hematologists, oncologists, and pathologists—often bewildered by an ever increasing flow of new scientific information, the practical application of which is often either not clearly disclosed or difficult to discern. Though the methods described in this volume are often used in combination for greater clinical impact, they are assembled here in 16 chapters grouped along five major themes: Cytogenetics; Polymerase Chain Reaction; Flow Cytometry; Cytochemistry and Immunohistochemistry; and Apoptosis and Cytokine Receptors.

Since the recognition by Tjio et al., in 1956 ***(1)***, that the human nuclear genome was contained in 46 rather than 48 chromosomes, the study of cytogenetics has evolved from the "dark ages" that preceded the development of the isolation and staining techniques necessary to identify individual chromosomes, to the current "molecular" era, in which detecting and locating specific genes, gene mutants, and gene rearrangements are possible through the use of DNA

probes. In Part 1, Drs. Sandberg, Baudis, and Hilgenfeld and their respective colleagues describe current methods for routine cytogenetics and fluorescence *in situ* hybridization (FISH), comparative genomic hybridization, and the recently introduced spectral karyotyping, respectively. They also review the clinical correlates of these techniques most useful for clinicians. Part 2 begins with Dr. Ward et al.'s review of those current methods in molecular biology and genetics that are most useful in the diagnosis and management of leukemia and lymphoma. This is followed by examples of applications of PCR for diagnosing T- and B-cell lymphomas, for detecting minimal residual leukemia, and for identification of the *NPM-ALK* fusion gene generated by the t(2;5) in non-Hodgkin's lymphoma, written by Drs. Sykes, Brisco, and Shurtleff and coauthors, respectively. Evolution from banding to PCR has expanded the precision and detection limits of cytogenetics analysis to approximately 10^{-5}. The exquisite detection level and discriminant power of the newer techniques have propelled the Human Genome Project, a multinational public and private endeavor from which enormous scientific and medical benefits are expected, including a clearer understanding of gene function and regulation, and the detection of prenatal and preclinical disease, since it provides both the framework and a database for development of new strategies for disease prevention and control. Clinical benefits from these techniques are quickly materializing, as demonstrated by recent reports addressing cancer diagnosis and treatment. Using DNA microarray technology to determine gene expression profiling, a recent study demonstrated molecular heterogeneity in diffuse large B-cell lymphoma, as defined by histological and immunohistochemistry studies ***(2)***, a finding that might have an impact on clinical prognosis. Indeed, though 76% of 42 previously untreated patients expressing genes characteristic of germinal center B-cells were alive five years from diagnosis, only 16% of patients expressing genes normally associated with in vitro activation of blood B-cells survived five years. Heterogeneity uncovered by molecular fingerprinting is likely to explain sharp differences in the response rates and prognosis of patients with otherwise homogeneous cancers, and to guide the development of targeted therapeutic strategies. Probably the first example of a designed gene function regulation strategy for cancer management is the use of the oral *Bcr-Abl* tyrosine kinase inhibitor STI571 in chronic myelogenous leukemia (CML). The hallmark of CML is the Philadelphia chromosome, characterized by replacement of parts of *c-Abl* (at *9q34*) with *Bcr* sequences (at *22q11*). The resulting *Bcr-Abl* fusion gene encodes a protein product with enhanced tyrosine kinase activity that is pivotal in transforming transfected cells and confers on them a proliferative advantage. In recent phase I/II clinical trials ***(3)***, 96% of 54 interferon-resistant CML patients treated with STI571 in daily

doses exceeding 300 mg achieved complete hematologic remissions, including 33% cytogenetic responses, with minimal toxicity.

In 1972, Herzenberg et al. designed the first fluorescence-activated cell sorter ***(4)***. Since then, flow cytometry (FCM) has evolved from being primarily an expensive research tool requiring high maintenance instruments, to a highly versatile technique with increasingly widespread uses in many biological and medical fields. As retraced by Dr. Marti et al., in Part 3, technical factors that contributed to this evolution include: the rapidly evolving hybridoma technology that has generated a myriad of monoclonal antibodies (MAbs) that recognize a large array of surface and nuclear antigens on hematologic and nonhematologic cells; the increasing understanding of lymphocyte subset differentiation and of the complexity of interacting interleukins; progress in fluorochrome technology that allows the simultaneous use of distinctly conjugated MAbs with different specificities, thus enabling discriminant analysis of complex cellular components within fluids and tissues, and refinement and simplification of the hardware and software involved. More recently, the widespread use of FCM has been propelled by its suitability for assessing CD4 count in HIV, CD34 in bone marrow transplantation, and for a host of other prevalent clinical applications, including phenotyping, cell cycle and chromosome analysis, cell subset count and sorting, and functional studies. Given its sensitivity and specificity, and the biologic significance of its findings, FCM is viewed today as an indispensable tool for diagnosing hematologic malignancies and for monitoring their progress and response to therapy. Drs. Braylan and colleague detail the methods utilized in their reference laboratory for detecting surface and nuclear antigens, and for cell cycle analysis. Drs. Mosiman and Goolsby review the present status of what is sometimes referred to as "molecular cytometry," a powerful technique that by combining FISH and FCM enables detection of DNA sequences of interest in cell population subsets defined by FCM. Finally, Dr. Vogt et al. address quantitative FCM issues that directly impact QA/QC and are finding increasing clinical usefulness as differing levels of antigen expression by normal and malignant cells are often associated with discriminant diagnostic and prognostic implications.

Cyto- or histochemistry, the technique of applying chemical stains to enhance microscopic differences among cells or tissues, originated with Francois Raspail in 1825 ***(5)***. Expansion of his initial observations and the systematic application of these "special stains" in the clinical setting were extremely slow. Furthermore, their lack of specificity and relatively low sensitivity reduce their usefulness so that they are now mostly used to confirm a particular interpretation, especially in surgical pathology, as described in Part 4 by Dr. Hanly, and led to the current immunohistochemistry era. This

latter approach exploits the specific binding between antibody and antigen, detected directly or indirectly by fluorescein- or enzyme-labeled primary or secondary antibodies, respectively. Demonstration of the power of this technique for distinguishing monoclonal from polyclonal disease in the clinical setting originated from our research laboratory. Using fluorescein-labeled antisera we identified a predominant monoclonal IgMκ cell population in histologically benign lymph node and involved lung tissue cryostat-frozen sections, in a patient with longstanding Sjögren's pseudolymphoma, 22 months before the lymphoma became clinically and histologically evident ***(6)***.

Though this initial approach was cumbersome and not easily adaptable to the clinical laboratory, developments over the subsequent two decades have led to the widespread adoption of immunohistochemistry, especially for the study of lymphomas. Contributing factors include: a broad range of MAbs directed against literally hundreds of antigens, the specificity of the binding reaction, and the advent of horseradish peroxidase-based techniques that allowed working with routinely processed tissues, thus enabling visualization of cellular constituents within well-preserved tissue sections. Yet, because antigens detected on malignant cells are also expressed by their normal, lineage-related cell counterparts immunohistochemistry assays are not disease-specific, with a few notable exceptions. Nevertheless, as Dr. Jaramillo et al. emphasizes, immunophenotyping helps detect evidence of malignancy, mainly through immunoglobulin light or heavy chain restriction, loss of pan-T or pan-B cell antigens, or aberrant cross-lineage antigen expression. Furthermore, distinct immunoarchitectural antigen expression patterns are characteristic of most lymphomas, a feature crucial in the differential diagnosis of these diseases. At present the concurrent assessment of immunohistochemistry, cytogenetics, and FCM has become the standard of practice in diagnosing and managing hematologic malignancies.

Part 5 presents overviews of apoptosis and of cytokine receptors analysis, written by Drs. Chiarugi and Zola, respectively. Apoptosis, or programmed cell death, is a normal physiologic process by which cells actively "commit suicide" and are removed by phagocytes, in sharp contrast to the process of necrosis that involves cell lysis followed by an inflammatory response. The discovery in 1985 of the *Bcl-2* was a milestone, for it provided the first example of an proto-oncogene that promotes clonal cell expansion by decreasing apoptosis rather than by promoting cell division ***(7)***. The best example of this is follicular B-cell non-Hodgkin's lymphoma, a slowly proliferative malignancy characterized by the presence of t(14:18). In this malignancy, the *Bcl-2* is translocated from chromosome 18 to 14, where it comes under the influence of immunoglobulin heavy chain gene-associated transcriptional elements that

lead to overproduction of Bcl-2 by malignant cells and, in turn, to decreased apoptosis. The process of apoptosis, regulated by gene-dependent counteracting influences that either promote or block cell death, has great potential relevance to cancer. Indeed, numerous types of cancers exhibit an overexpression of *Bcl-2*, several homologs of the *Bcl-2* family have been discovered in certain tumor-associated viruses, and it is now clear that gamma irradiation, and most if not all chemotherapeutic drugs, initiate apoptosis. Thus, novel anticancer strategies can be devised to modulate the intracytoplasmic concentrations of pro- and anti-apoptotic members of the *Bcl-2* family in favor of the former. Cytokines are a large and complex family of soluble protein mediators secreted by leukocytes, and they affect the growth, activation, or function of cells of the immune, hematopoietic, and other systems through cell surface receptors. Though our understanding of their role in malignancy is still fragmentary, their value in cancer management is underscored by their successful clinical applications. In a supporting role, colony-stimulating factors and, to a lesser extent, IL11 have become the standard of care for shortening the severity and duration of postchemotherapy leukopenia and thrombocytopenia, respectively. Additionally, certain cytokines exhibit anticancer activity, as is the case of IFN-α that induces the highest complete cytogenetic remission rates in CML when given concomitantly with cytosine arabinoside. Most patients who remain in continuous cytogenetic remission for at least one year appear to be free of residual disease, as judged by RT-PCR-negative *Bcr-Abl*. Finally, cell surface cytokine receptors can be used for targeted therapy, as demonstrated by DAB_{389}IL-2, the first such agent approved by the FDA for clinical use. This agent is an IL-2 receptor-specific ligand fusion-protein produced by expression of a recombinant fusion gene in *Escherichia coli*. The fusion product consists of the first 389 residues of diphtheria toxin, containing the catalytic and transmembrane domains, fused to human IL-2. Upon binding to putative high affinity IL-2 receptors, preferentially expressed by cutaneous T-cell (CTCL) and other lymphomas, the catalytic domain is endocytosed and released into the cytosol, inducing protein synthesis inhibition and, ultimately, to cell death. In recent phase I clinical trials (***8***), 37% of 35 heavily pretreated patients with CTCL responded to DAB_{389}IL-2 doses ranging from 3 mg/kg/d to a maximum tolerated dose of 27 mg/kg/d, including 14% complete remissions. Several other targeted fusion-protein toxins and immunotoxins (antibody-toxin conjugates) have also demonstrated significant anticancer activity in early clinical trials. However, challenging pharmacological issues and substantial toxicity limit the therapeutic index and efficacy of this group of agents.

These are exciting times in which accelerating advances in bioscience and biotechnology are enabling us to address fundamental questions about the

nature, origin, and growth regulation of cancer. They provide increasingly discriminant tools for the detection of neoplasia at the molecular level, and have increased the feasibility of developing more selective anticancer agents and of designing rational strategies for its control and eradication. Although most initial steps involving these strategies have met with limited success, they do represent a welcome departure from traditional semi-empirical drug development and testing by solidly grounding cancer therapy in our evolving knowledge of cancer genetics, biology, and growth regulation. It is hoped that the information contained in *Hematologic Malignancies: Methods and Techniques* will not only prove helpful to all research workers and health care providers managing patients with hematologic malignancies, but also serve to entice a new generation of young scientists to consider a career in cancer research.

Guy B. Faguet, MD

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1

CYTOGENETICS

1

Cytogenetics Analysis

Avery A. Sandberg and Zhong Chen

1. Introduction

1.1. Principles

The establishment of reliable and meaningful chromosomal (cytogenetic, karyotypic) changes in hematological disorders, primarily the leukemias and lymphomas, must be based on the examination of the involved cells or tissues. Thus, in the case of the leukemias bone marrow (BM) aspirations yield optimal results in the preponderant number of patients, whereas in the lymphomas affected tissues, usually lymph nodes, are the best source of cells carrying cytogenetic anomalies. Generally, BM is not a good source of cells for cytogenetic analysis in lymphoma. Not only is the marrow often not affected by the lymphoma, but also when it is, the number of abnormal cells is relatively small and/or the abnormal cells are not in division and, hence, do not yield a sufficient number of metaphases for cytogenetic analysis. In some situations, blood cells can be utilized as a source of metaphases affected by karyotypic changes, e.g., in cases with about 10% immature cells in the peripheral blood (PB), in chronic lymphocytic leukemia (CLL), in cases where the marrow is fibrotic or extremely hypocellular, or in determining the presence of Ph+ cells in established cases of chronic myelocytic leukemia (CML) ***(1,2)***.

Paramount for a successful cytogenetic study is the presence of metaphases suitable for analysis. In the normal BM, a significant number of dividing cells, and hence metaphases, are usually present in sufficient number for cytogenetic analysis without having to resort to culture or lengthy incubation. However, in some leukemias the number of dividing cells (especially the leukemic ones) is very low and, hence, incubation of the marrow specimen for a number of days (2–5 d) may be necessary to generate a significant number of metaphases for

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cytogenetic study. The statements just made apply in particular to acute promyelocytic leukemia (APL) and following chemotherapy and/or radiation therapy for the leukemia ***(1,2)***.

In cases where cytogenetic analysis reveals only abnormal metaphases, especially those with a balanced translocation, it may be necessary to rule out a constitutional chromosomal anomaly. This is best established through the cytogenetic examination of phytohemagglutinin (PHA) stimulated lymphocytes of the PB.

A number of mitogenic agents capable of stimulating lymphoid or, less frequently, myeloid cells have been introduced over the years. Outstanding among these has been PHA capable of stimulating the growth and division of lymphocytes of T-cell origin. However, PHA is not routinely added to BM or PB cultures in acute leukemias because PHA may interfere with the evaluation of spontaneously dividing malignant cells.

The quality of chromosome preparations has been significantly improved with some new techniques, such as the use of amethopterin for cell synchronization, the use of short exposures to mitosis-arresting agents, the use of DNA-binding agents to elongate chromosome ***(3,4)***, improved staining procedures, and the use of conditioned culture medium containing hematopoietic growth factors [e.g., GCT (giant cell tumor)-conditioned medium primarily for myeloid disorders ***(4,5)*** and PHA/IL-2 (interleukin-2) for both B- or T-cell lymphoid diseases ***(6,7)***].

The rate of successful cytogenetic analysis varies with the specific type of disease, and is also related to the adjustment of variables in each laboratory, such as serum concentration, medium pH, and cell concentration.

1.2. Clinical Applications

The common and recurrent chromosome changes seen in the leukemias and lymphomas are shown in **Tables 1–3** and **Figs. 1** and **2**.

CML is a pluripotent stem cell disorder characterized cytogenetically by the Philadelphia chromosome (Ph), the first consistent abnormality observed in a human cancer. The Ph arises from a reciprocal translocation, t(9;22)(q34;q11) ***(8)***. It is characterized molecularly by the fusion of parts of the *C-ABL* gene (at 9q34) and the *BCR* gene (at 22q11), generating an abnormal *BCR/ABL* fusion gene ***(9)***. Cytogenetically, more than 85% of patients with CML are found to have the Ph in the CML cells, even during remission, unlike the Ph in acute leukemia, which is not seen during complete remission. When CML progresses, additional changes, such as +8, +Ph, i(17q), +19, and +21 are noted in 75–80% of cases. These changes may precede hematologic progression by 2–6 mo or occur at the blast phase; therefore, they are valuable prognostic indices. However, there is no evidence that these additional changes correlate with response

Table 1
Common Chromosome Changes in Acute Nonlymphocytic Leukemia (ANLL) *(1,12)*

der(1;7)(q10;p10)[a]	t(9;22)(q34;q11) M1(M2)
t(1;22)(p13;q13) M7	t(11;V)(q23;V)[b] M5(M4)
ins(3;3)(q26;q21q26)[a,d] M1(M7)	del(11)(q23) M5(M4)
inv(3)(q21q26)[a,d] M1(M7)	+11
t(3;3)(q21;q26)[a,d] M1(M7)	del(12)(p11p13)[a]
t(3;21)(q26;q22)[a]	+13[a]
+4 M2, M4	+14[a]
–5 or del(5)(q12–13 or q31–35)[a] M1–M4	t(15;17)(q22;q11–21) M3
+6	del(16)(q22)[c] M4EO
t(6;9)(p23;q34)[a] M2(M4) (basophilia)	inv(16)(p13q22)[c] M4EO
–7 or del(7)(q22)[a] M1–M5	t(10;16)(p13;q22)[c] M4EO
+8[a]	t(16;21)(p11;q22)
t(8;16)(p11;p13) M5b (erythrophagocytosis)	i(17)(q10)[a]
t(8;21)(q22;q22) M2 (Auer rods+)	+19
+9	del(20)(q11–13)[a]
del(9)(q22)	+21
	idic(X)(q13)[a]

[a]Change also seen in myelodysplastic syndromes.
[b]V = chromosomes 6, 9, 17, and 19.
[c]Associated with marrow eosinophilia.
[d]Associated with platelet and/or megakaryocytic anomalies.
Where appropriate, the type of ANLL or other information associated with a particular chromosome change is also shown.

to presently used therapy during the acute phase of CML. Clinically, treatment strategies for CML should include, in addition to the hematologic criteria, the cytogenetic findings and the molecular genetic criteria of the *BCR/ABL* fusion gene obtained using Southern blotting or polymerase chain reaction (PCR) techniques.

Myelodysplastic syndromes (MDS) are a heterogeneous group of clonal hematopoietic stem cell disorders characterized by dysplastic and ineffective hematopoiesis and a high risk of transformation to ANLL. Clonal chromosomal abnormalities can be detected in 40–70% of MDS patients at presentation (*see* **Table 4**). Additional aberrations may evolve during the course of MDS and appear to portend its transformation to leukemia. To confirm the diagnosis of MDS, morphologic examination of BM aspirate and cytogenetic analysis should be performed. Moreover, the chromosomal findings have been shown to be an independent prognostic indicator second only to the French-American-British (FAB) classification subtype as a predictor of progression to leukemia and survival (*see* **Table 5**).

Table 2
Common Chromosome Changes in B-Lineage Acute Lymphocytic Leukemia (ALL) *(1,12)*

t(1;9)(q23;p13)	Pre-B-cell
t(2;8)(p12;q24)	L3 (B-cell)
t(4;11)(q21;q23)	Biphenotypic, early B-precursor
t(5;14)(q31;q32)	
del(6)(q13–14 or q21–27)	
t(8;14)(q24;q32)	L3 (B-cell)
t(8;22)(q24;q11)	L3 (B-cell)
del(9)(p13–22)	
t(9;22)(q34;q11)	B-lineage
del(11)(q14–23)	
t(11;19)(q23;p13)	Mixed, biphenotypic
t(12;V)(p12;V)[a]	B-lineage
t(14;19)(q32;q13)	
t(14;22)(q32;q11)	

[a]V = Chromosomes 7, 9, and 17.

Table 3
Common Chromosome Changes in B-Cell Non-Hodgkin Lymphoma (NHL) *(1,12)*

Chromosome changes	Histology
t(2;3)(p12;q27)	Diffuse large cell
t(2;8)(p12;q24)	(Burkitt) small noncleaved cell
t(3;14)(q27;q32)	Diffuse large cell
t(3;22)(q27;q11)	Diffuse large cell
+3	Follicular large cell, immunoblastic
del(6q)	Follicular small cleaved cell
t(8;14)(q24;q32)	(Burkitt) small noncleaved
t(8;22)(q24;q11)	(Burkitt) small noncleaved
t(11;14)(q13;q32)	Centrocytic (variable zone) with $CD5^+$ cells
+12	Diffuse small cell
t(14;18)(q32;q21)	Mixed, small cleaved, and large cell follicular
t(18;22)(q21;q11)	Follicular

The acute leukemias, which are classified either as lymphoblastic (ALL) or nonlymphocytic (ANLL), result from neoplastic transformation of uncommitted or partially committed hematopoietic stem cells. About two thirds of ANLL and ALL patients have recognizable clonal chromosomal anomalies. These

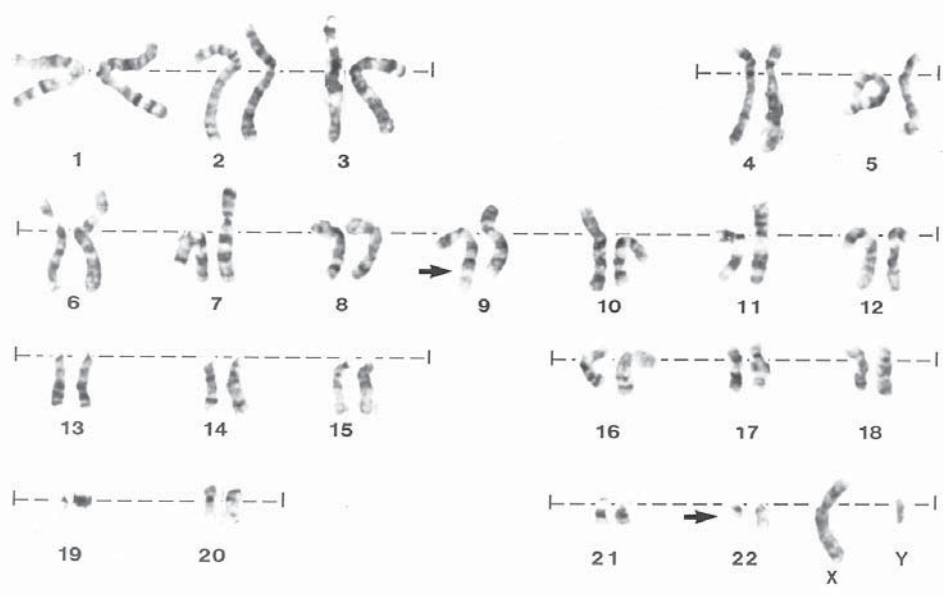

Fig. 1. G-banded karyotype of a marrow cell showing the Philadelphia (Ph) translocation, t(9;22)(q34;q11) (arrows point to breakpoints). This was the only change present in the affected cells of this case with CML.

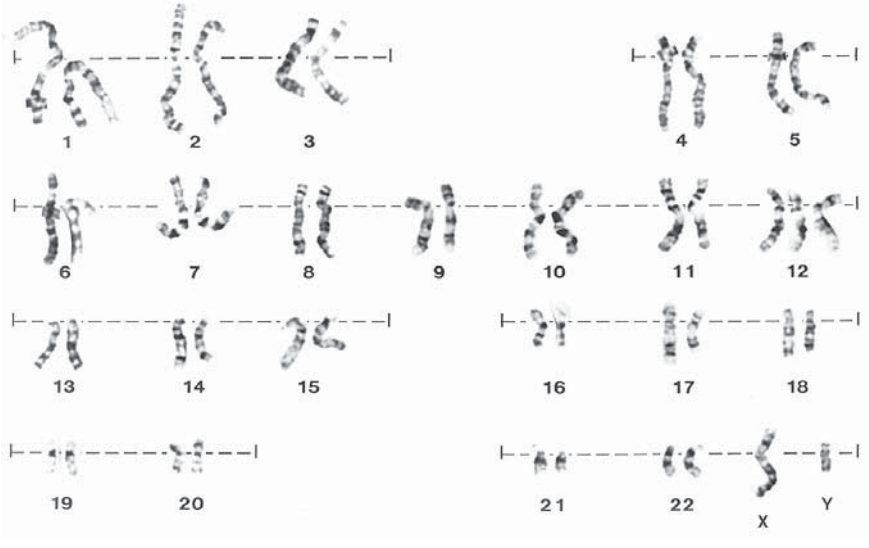

Fig. 2. G-banded karyotype showing trisomy 12 (+12) as the only change in a case of CLL. This change (+12) is seen in a significant number of CLL cases and is usually associated with a poor prognosis.

Table 4
Frequency of Chromosomal Changes and Evolution to ANLL in Myelodysplasia

FAB subtypes and distribution	Evolution to ANLL	Chromosomal changes	mo
Refractory anemia (30%)	11%	48%	37
Refractory anemia			
With ring sideroblasts (18%)	5%	12%	49
With excess blasts (25%)	25%	57%	9
With excess blasts & transformation (12%)	50%	93%	6
Chronic myelomonocytic leukemia (15%)	15%	29%	22

Table 5
Prognosis in Myelodysplastic Syndromes According to Cytogenetic Findings

Prognostic category	Karyotype	Median survival (mo)
Good	Normal	>24
	Deletion 5q	
Intermediate	Trisomy 8	18
Poor	Monosomy 7	
	Deletion 7q	
	Isochromosome 17q	<12
	Deletion 20q	
	Complex changes	

may fall into a specific category that characterizes unique clinical and cytogenetic entities. Survival, as a function of cytogenetic findings in ANLL and ALL, is shown in **Table 6**. The determination of the chromosomal changes in acute leukemia serves a number of practical purposes, for example, the establishment of the exact diagnosis, prediction of prognosis, and as a guide to the treatment and monitoring phases of therapy or BM transplantation, as well as some basic purposes, such as supplying the molecular biologist with possible information on the location or nature of the genes affected by translocations, deletions, and inversions. A case in point is the t(15;17)(q22;q21), seen in APL, which has been shown to affect a gene related to the α-retinoic acid receptor. This has led to the use of retinoic acid in the therapy of APL with remarkable results.

More than 90% of non-Hodgkin lymphomas (NHL) have clonal chromosomal changes; t(8;14)(q24;q32), t(8;22)(q24;q11) and t(2;8)(p12;q24) have

Table 6
Chromosomal Abnormalities and Survival in ANLL and ALL

Chromosomal abnormality	Median survival (mo)
ANLL	
Rearrangements of 16q22	18
Translocation (8;21)(q22;q22) (*see* **Fig. 3**)	14
Normal karyotype	10
Abnormal 11q	8
Abnormal 5 and/or 7	3
Translocation (15;17)(q22;q21)[a] (*see* **Fig. 4**)	*a*
ALL	
More than 50 chromosomes	58
Deletion 6 (q15q21)	30
Normal karyotype	29
Translocation (9;22)(q34;q11)	12
Less than 46 chromosomes	12
Rearrangements of 14q32	8
Translocation (4;11)(q21;q23)	7
Rearrangements	5

[a]Survival for patients with a 15;17 translocation is markedly improved with aggressive treatment and trans-alpha retinoic acid therapy compared with the median survival previously reported (2 mo)

been found in 75–80%, 10–17%, and 5–8% of Burkitt lymphomas (BL) of both African and non-African origin, respectively ***(10)***. Molecularly, fusion of the *MYC* gene to immunoglobulin genes has been identified in all BL cases. In non-Burkitt NHL, a 14q+ marker characterizes about 50% of the cases. Many of the nonrandom anomalies correlate with histology and immunologic phenotype, such as t(14;18)(q32;q21) with follicular (nodular) B-cell lymphomas, del(6q) with large-cell lymphomas, and t(8;14)(q24;q32) with either small, noncleaved cell or diffuse large-cell lymphomas.

Approximately 50% of CLL patients have chromosomal abnormalities, the most common of which are trisomy 12, 14q+, 13q, and 11q abnormalities. An abnormal karyotype is a poor prognostic sign in CLL, and trisomy 12 and possibly 14q+ are the least favorable abnormalities. Three factors are of importance in CLL: lymphocyte doubling time, diffuse lymphocyte infiltration of BM and lymph nodes, and the chromosomal pattern. Combining these three factors with the current clinical staging of CLL may optimize therapeutic decisions.

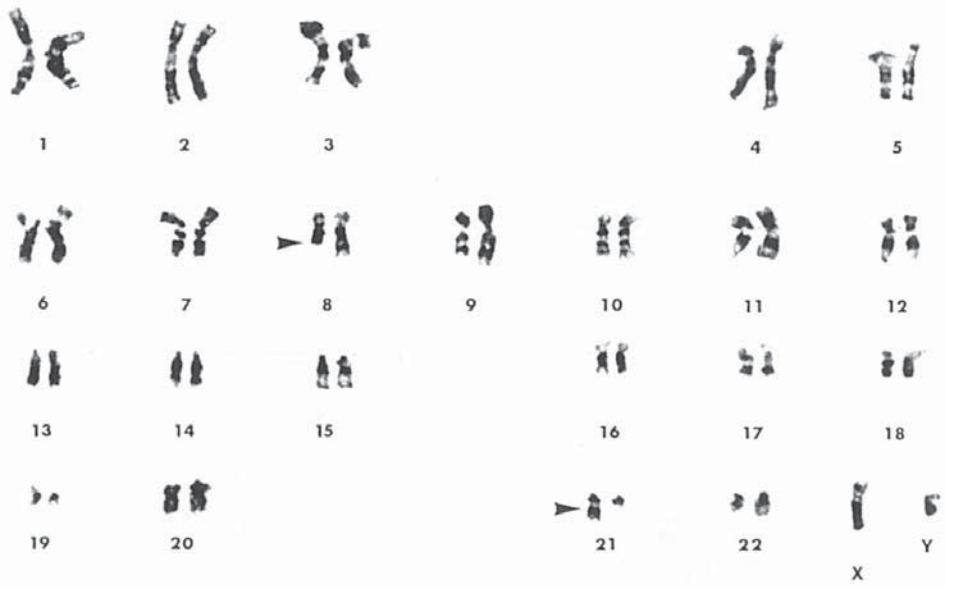

Fig. 3. G-banded karyotype with the translocation (8;21)(q22;q22) as the only anomaly. This change is seen almost exclusively in M2 type of ANLL associated with a relatively good prognosis, Auer bodies in the cells, and a high remission rate.

2. Materials

2.1. Specimens

1. BM aspirate or bone core biopsy: One to 3 mL of BM should be aseptically aspirated into a sodium-heparinized syringe and transferred to a sterile sodium vacutainer tube. The specimen can be transported with or without culture medium (RPMI 1640 + 5–10% fetal calf serum [FCS] + 1% penicillin[pen]/streptomycin[strep]). If the specimen cannot be delivered immediately, it may be stored at room temperature or in a refrigerator overnight. Do not freeze the specimen. Cell viability drops off sharply by 72 h after collection. If a marrow aspirate cannot be achieved, a BM biopsy may be accepted for cytogenetic analysis.
2. Peripheral blood: Five to 10 mL of PB should be aseptically collected and transferred, transported, and preserved in the same way as for a BM specimen.
3. Lymph node and spleen: Lymph node and spleen biopsies or samples should be collected aseptically and transferred to a sterile sodium vacutainer tube containing culture medium (RPMI 1640 + 5–10% FCS + 1% penicillin/streptomycin). The specimen should be transported and preserved in the same manner as for BM specimen.

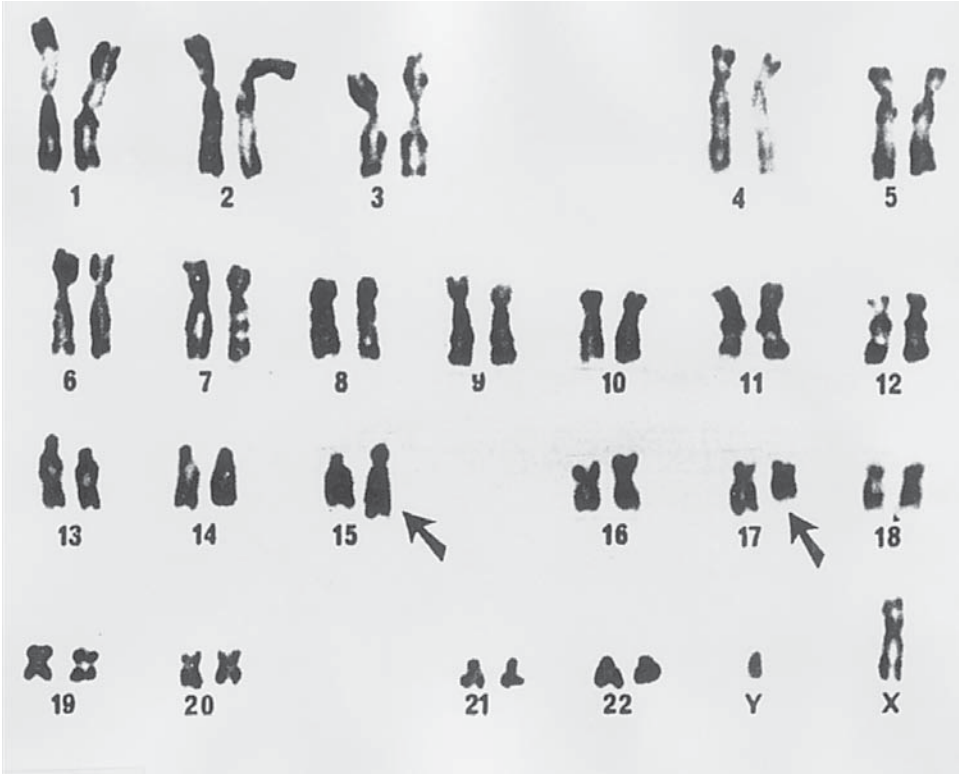

Fig. 4. R-banded karyotype of a cell from a patient with APL (M3) containing the translocation (15;17)(q22;q11–21) as the only chromosome change. This translocation is characteristic of APL (M3).

2.2. Reagents and Instruments

1. RPMI 1640/MEM Alpha/FBS complete media: 100 mL RPMI 1640, 70 mL MEM alpha, 30 mL fetal bovine serum (15%), 2 mL 3% L-glutamine (1%), 2 mL penicillin/streptomycin (1%) (10,000 units pen/mL; 10,000 µg strep/mL).
2. Colcemid (Gibco Karyomax colcemid solution—10 µg/mL):
   a. 1/20 Solution: Dilute 10 mL of the stock solution with 10 mL of sterile deionized water to a final concentration of 5 µg/mL). Store at 4°C.
   b. 1/200 Colcemid solution: Dilute 5 mL of 1/20 colcemid with 45 mL of sterile deionized water to a final concentration of 0.5 µg/mL). Store at 4°C.
3. Potassium chloride—0.068 *M*:
   a. Stock solution: 5.6 g KCl in 100 mL of deionized water.
   b. Working solution: 10 mL KCl of stock solution in 100 mL of deionized water. Prewarm to 37°C before use.

4. Fixative: Freshly prepared 3 parts of absolute methanol to 1 part of glacial acetic acid, then chill in freezer.
5. Giant cell tumor cell line: Available from the American Type Culture Collection (ATCC). Maintain cell line in culture and collect supernatant, filter and freeze at –10°C to –20°C. Bring up new vial after current cells have been passaged 20 times.
6. PHA (Gibco): 5 ml added to the lyophilized reagent. Store at –10°C to –20°C.
7. Interleukin-2 (Il-2) (Boehringer): 200 µL (working solution). Store at –10°C to –20°C.
8. Ethidium bromide (EB): 10X stock solution (10 mg/mL) diluted with deionized water to 1X working solution (1 mg/mL).
9. pH 6.8 buffer: Dissolve one Gurr's pH 6.8 buffer tablet in 1000 mL of distilled water. Store at room temperature.
10. pH 7.0 buffer: Use one Gurr's 7.2 buffer tablet and 9 g of NaCl. Make up to 1000 mL with distilled water and store at room temperature.
11. Giemsa stain (Harleco or Gurr's): Mix 2.5 mL of Harleco Giemsa and 0.5 mL of acetone with 40.0 mL of pH 6.8 buffer. Make up new after 2–4 h of previous stain solution.
12. Stock trypsin solution (Difco): Dissolve 1.25 g of trypsin 1:250 in 200 mL of distilled water. Dispense 4 mL into 12 × 75 mm tubes and store frozen.
13. Working trypsin solution: 0.025%. Mix 2.0 mL of stock trypsin solution with 40.0 mL of pH 7.0 buffer solution. Make up just before use in the morning (which is satisfactory for rest of the day).
14. T-25 flasks.
15. 15 ml centrifuge tubes.
16. CO2 incubator.
17. Disposable pipets.
18. Oven.
19. Slides.
20. Biohazard hood.
21. Coulter counter.
22. Conventional light microscope with phase.
23. 150 ml sterile orange-top bottles.
24. Centrifuge.
25. Large bottle repeat pipettor for fix.
26. Incubator.
27. Slide warmer.
28. Pro-Texx mounting medium.
29. Waterbath.
30. Isopettes.
31. Serological pipets: 5 mL and 10 mL.

3. Methods

3.1 BM and PB specimens

3.1.1. Enumeration of Cells—Coulter Counter

Using Isopette vials, make a dilution of each specimen and establish white cell count (WCC) using the Coulter counter (*see* **Note 1**). Determine the amount of sample per culture. Optimum concentration is 10×10^6 cells/10 mL culture.

3.1.2. Quick Differentials

Quick differentials are used when the WCC is suspicious or when the Coulter counter is not working (*see* **Note 2**). The following protocol is for use with the Hemacolor stain set (EM Diagnostic Systems).

1. Working under the biohazard hood, place 1 drop of BM of PB onto a slide. Using another slide, spread the sample evenly across the slide. Allow to dry.
2. Immerse the slide in solution 1 (fixative solution) five times, 1 s each time. Allow to drain.
3. Immerse the slide in solution 2 (phosphate-buffered eosin solution) for 1 s, remove and hold in air for 1 s. Repeat three times, drain.
4. Immerse the slide in solution 3 (phosphate-buffered thiazine solution) four to five times, holding in the air for 1 s between immersions.
5. Rinse with distilled water. Allow to dry.

3.1.3. Checking Specimen Adequacy

Specimen cellular adequacy should be made on the culture sheet (*see* **Note 3**). To get an estimate of hypo, hyper or average cellularity using the thin side of the smear, a low, average, increased, and high WCC would expect approx 40, approx 100, approx 200, and approx 400 white cells/field. Based on these estimates, the amount of sample to be added to each slide can be derived, as follows:

WCC (K)	Quick differential	Amount (mL)
<2	Low	1.0
4–10	Average	0.5
20	Increased	0.25
>40	High	0.1

3.1.4. Culture

Set up the specimen, according to the amount available, with specimen volume adjusted so that the final concentration of white cells is approximately 1×10^6 mL. In specimens with a cell count falling within the normal range, 0.5–0.75 mL of the sample will yield the correct cell concentration. All cultures should be set up in T-25 flasks. Culture priority (*see* **Note 4**), based on the clinical information provided for each patient, is described as follows:

1. For myeloid disorders in patients >2 yr old
   a. 24 h overnight with 1/200 colcemid: 10 mL GCT media + BM specimen. Add 0.1 mL of colcemid (1/200) to culture at 5 p.m. on day culture is set up. The next morning, begin harvest immediately.
   b. 48 h culture: 10 mL of GCT media + BM specimen. Harvest at 48 h.
   c. Backup culture: 10 mL of GCT media + double amount of BM specimen. Harvest at 4–5 d, if needed.
2. For lymphoid disorders, anemia of unknown causes, unclassified leukemia, and leukemias in patients <2 yr old:
   a. 24 h overnight with 1/200 colcemid: 10 mL of GCT media + BM specimen. Add 0.1 mL of colcemid (1/200) to culture at 5 p.m. on day culture is set up. The next morning, begin harvest immediately.
   b. 72 h PHA/IL2: 10 ml of GCT media + marrow specimen + 0.1 ml PHA + 0.1 mL IL-2. Add 0.1 mL of 1/200 colcemid at 5 p.m. the day before harvest.
   c. 48 h culture: 10 mL of GCT media + BM specimen. Harvest at 48 h.
   d. Backup culture: 10 mL of GCT media + double amount of BM specimen. Harvest at 4–5 d, if needed.

3.1.5. Harvest

1. For all cultures, be sure that 0.1 mL of 1/200 colcemid is added at 5 p.m. the day before harvest.
2. On the morning of harvest, add 0.1 mL of 1 mg/mL EB to all cultures being harvested. Incubate for 60 min.
3. Empty flasks contents into 15 mL conical centrifuge tubes (one tube for each culture), transferring label from each flask to its tube, and spin down at 1200 rpm in a tabletop centrifuge for 8 min.
4. Aspirate off supernatant; gently resuspend pellet by tapping.
5. Add 10 mL of prewarmed 0.068 *M* KCl to pellet and gently mix with a Pasteur pipet (*see* **Note 5**). Add 0.125 mL of 1/20 colcemid. Gently mix. Colcemid on the backup culture may be increased five times if no metaphases were seen on the two previous cultures.
6. Allow to stand at 37°C for 30 min. Add 1.0 mL of 3:1 prefix. Gently mix with Pasteur pipet.
7. Spin down for 8 min at 1200 rpm in a tabletop centrifuge. Aspirate off supernatant. Add 10 mL of 3:1 fix with a 10-mL pipet. Mix with a Pasteur pipet thoroughly

(break up clumps if possible, *see* **Note 6**). Allow to stand at 0°C (i.e., in ice or in freezer) for a minimum of 10 min. The specimen can be stored in the refrigerator at this point, or after the following two fixes, if more time is needed for specimens, or if the harvest cannot be completed on the same day. Be sure the fix stays cold.

8. Spin down at 1200 rpm for 8 min in a tabletop centrifuge, aspirate off supernatant. Gently resuspend pellet by tapping before adding 5 mL of 3 : 1 fix. Allow to stand at 0°C (i.e., in ice or in freezer) for a minimum of 10 min. Be sure the fix stays cold.
9. Spin at 1200 rpm in a tabletop centrifuge for 8 min. Aspirate off supernatant and gently resuspend pellet in remaining fix. Then add 5 mL of cold fresh fix. Allow to stand at 0°C (i.e., in ice or in freezer) for a minimum of 10 min. Be sure the fix stays cold.
10. Spin at 1200 rpm in a tabletop centrifuge for 8 min. Aspirate off supernatant; get close to pellet. Resuspend gently in remaining fix. Add fresh, cold fix, drop by drop, to desired dilution. Store suspensions at 4°C in refrigerator until slide making.

3.1.6. Slide Preparation

The basis for an informative chromosome analysis often resides in the quality of the slide preparation (*see* **Note 7**). An optimal slide should have well-spread, but complete, metaphases with a minimum of overlapping chromosomes and no cytoplasmic background around the metaphases. Because the humidity and the sensitivity of cells to the hypotonic solution are variable, the ability to adjust the slide-making technique to the prevailing conditions is crucial. Several techniques for making slides are suggested as follows.

1. Use wet slide with bead of water on it or a dry slide.
2. Drop specimen material from a height of 1/2–2 in. (you may blow lightly to aid spreading).
3. (If needed:) A couple of drops of acetic acid to cell suspension may aid in spreading. Flaming may also improve spreading, but may interfere with banding.
4. Check under phase microscope. Add more fix or respin and resuspend to achieve proper cell dilution.
5. Put three good quality slides on a 60°C slide warmer to age overnight before banding. Alternatively, the slides may be heated for 20 min in a 90°C oven. Prior to banding, expose the slides to UV light in the hood for 45 s.
6. Add 3 mL of fixative to the remaining cell suspension; cap and store tube in the refrigerator. After analysis is complete, and if the results are normal, the cells may be discarded. If the results are abnormal, the cells may be stored in a freezer at –20°C for many years for future use.

3.1.7. Slide Making and Staining (GTG Banding)

1. Treat slides with 0.025 of trypsin solution at pH 7.0. Time of trypsin treatment varies from one preparation to another. Also, the time increases with age of slides (*see* **Note 8**).

2. Rinse in pH 7.0 buffer for 1–2 s.
3. Rinse in second pH 7.0 buffer solution for 1–2 s.
4. Stain in freshly prepared Giemsa for 60–120 s. (this jar should remain capped at all times).
5. Rinse the slides in distilled water.
6. Blot the slides gently using bibulous paper until completely dry.
7. Adjust final trypsin and staining times based on the initial microscopic quality of initial slides.

3.2. Modified Procedures for Lymph Node and Spleen Specimens

3.2.1. Specimen Pretreatment

1. Transfer the specimen to a Petri dish.
2. Add a few drops of culture medium.
3. Cut the specimen into very small pieces with sterile scissors until a cell suspension is obtained.

3.2.2. Culture, Harvest, Slide Reparation, and Banding/Staining

Follow steps described in **Subheading 3.1.** for BM and PB specimens (lymphoid disorders).

3.3. Analysis

1. In general, a minimum of 20 cells must be counted and analyzed for each case, such that a rearrangement affecting one band of any chromosome can be detected in any given cell. The cells must be selected to represent at least two culture conditions. The individual morphology and band-by-band structure of each chromosome should be checked in each cell analyzed. Difficult cases may have to be photographed and analyzed on a print.
2. When possible, select cells with at least 300-band resolution and few overlaps.
3. Determination must be made whether one or more clones exist. A clone is defined as two or more cells having the same rearrangements or additional chromosomes, or three cells with the same monosomy ***(11)***. However, one cell with a normal karyotype is considered adequate evidence to indicate the presence of a normal cell line.
4. When a single abnormal metaphase is found in the analysis of the first 20 cells, an additional 20 cells may be screened. This allows identification of a 10% abnormal cell line with 87% confidence. Chronic lymphoproliferative diseases tend to have a low mitotic rate, and a single abnormal cell may represent the only dividing malignant cell observed in a particular sample (*see* **Note 9**).
5. When a patient with previous abnormal results is analyzed and only normal cells are seen, an additional 10 cells may be screened for the previous abnormality. This allows detection of 10% mosaicism with 95% confidence.
6. In general, all cases should be shared by two or more technologists. Any study that has no metaphases or no analyzable metaphases should have an experienced technologist as the final evaluator (*see* **Note 10**).

4. Notes

1. Coulter counter background readings must be kept low (less than 100). Flush the system between readings to avoid problems.
2. Coulter counter readings should take 13 (+/–1 s). If longer, this could mean the system is clogged. If shorter, the mercury level may be low.
3. Clots may form in the specimen or culture due to inadequate heparin, breakdown of heparin, or abnormalities in the clotting mechanism of the patient. These clots may be broken up by the aspiration through a needle or pipet and/or minced. Clots can trap cells and interfere with the harvesting procedure.
4. Mislabeling is a common source of error. This can be prevented by labeling and handling only one specimen at a time and by double checking labels and numbers.
5. Avoid using the same pipet during harvest on two different patients.
6. Cell clumps may form during harvest due to failure to resuspend pellet thoroughly prior to fixation, failure to mix cell suspension while adding fixative, or abundance of erythrocytes. The formation of these clumps may be prevented by resuspending the pellet thoroughly after hypotonic treatment. Add up to 1 mL of fixative dropwise while gently tapping the tube. For tubes containing a large proportion of erythrocytes, bring the volume of fixative to 10–15 mL immediately. It may be necessary to mix the cell suspension gently with a Pasteur pipet or to split the sample into two tubes, which can be combined after one to two changes of fixative.
7. Poor spreading of metaphase cells and the presence of cytoplasm often occur during preparation due to poor fixation and/or poor swelling of cells during hypotonic treatment. To overcome these problems, the following should be performed: repeating fixation three to four more times, increasing glacial acetic acid concentration up to 50%, dropping the cell suspension from a greater height, increasing the angle at which the slide is held, and increasing the humidity when slides are drying. An increase in humidity may result in poor fixation.
8. Poor G-bands may be a clue to the presence of cytoplasm, age of slides, and high humidity. The banding results can be improved by increasing the time in peroxide and /or trypsin, preparing fresh slides (as in the preceding solution) to improve the quality of metaphase spreads (slides older than 1 mo may give inconsistent results). The trypsin treatment may need to be lengthened), and placing slides in a drying oven or on a slide warmer (60°C) for several hours (perform banding immediately after removal).
9. BM is a difficult specimen from which to obtain good material for analysis. In general, the more malignant the cells, the poorer the quality of chromosome preparations. It is important that the poor quality cells are not skipped over in favor of the "prettier" cells, as this may bias the results.
10. With occasional specimens, due to the patient's disease process, there may not be any or enough mitotic cells to analyze. This fact in itself is informative to the physician.

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2

FISH Analysis

Avery A. Sandberg and Zhong Chen

1. Introduction

1.1. History and Principles

In situ hybridization of specific DNA or RNA sequences to cellular targets was developed over 20 yr ago ***(1,2)***. The early techniques employed isotopically labeled probes and subsequent autoradiographic detection using a photographic emulsion overlying the metaphase chromosomes, nuclei, or whole cells. However, autoradiography requires long exposure periods, and is not practical for clinical application. In the late 1970s, nonisotopic methods of nucleic acid labeling were developed. The subsequent improvements in the detection of reporter molecules using immunocytochemistry and immunofluorescence, in conjunction with advances in fluorescence microscopy and image analysis, have made the technique safer, faster and reliable.

During the past few years fluorescence *in situ* hybridization (FISH) has emerged as an extremely important tool for both basic and clinical research and application. This chapter focuses on FISH with DNA probes only. FISH is a technique that allows DNA sequences to be detected on metaphase chromosomes and interphase nuclei in tissue sections by using DNA probes specific for entire chromosomes or single unique sequences/genes. The steps of a FISH procedure are summarized in **Fig. 1**. In general, a specimen is treated with heat and formamide to denature the double-stranded DNA to become single stranded. The target DNA is then available for binding to a DNA probe with a complementary sequence that is also similarly denatured and single stranded. The probe and target DNA then hybridize to each other in a duplex based on complementary base pairing. The probe DNA is tagged with a hapten (such as biotin or digoxigenin) or is directly labeled with a fluorescent dye. Detection

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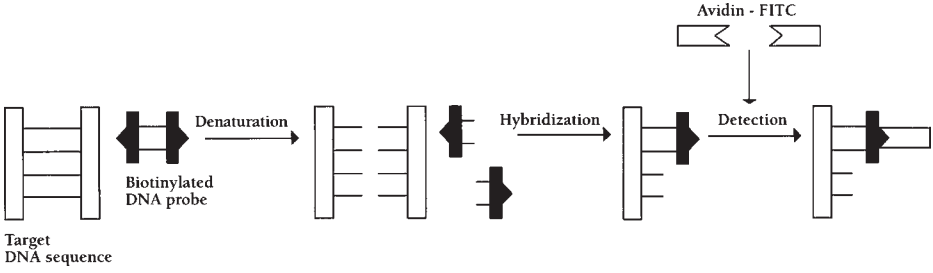

Fig. 1. Schematic presentation of some of the essential steps involved in FISH analysis.

of the hapten can be achieved with the application of an antibody tagged with a fluorescent dye (such as fluorescein, rhodamine, or Texas Red). Hybridization signals on a target material can be visualized through the use of a fluorescence microscope *(3)*.

1.2. Comparison of FISH vs Conventional Cytogenetics

A large number of acquired chromosome changes have been reported in hematological malignancies that correlate with specific clinical, morphologic, and immunophenotypic features *(4,5)*. Cytogenetic analysis is, therefore, a powerful tool in the assessment of these conditions. However, cytogenetic analysis alone is sometimes not sufficient to detect the chromosomal changes due to the fact that cytogenetic analysis can be performed only on dividing cells and the limitation of cytogenetic methods in some cases in which the abnormality is not visible with a conventional optic microscope.

FISH studies of the organization and function of chromosomal nucleic acid sequences have made it possible to gain information about chromosome changes in cells that are not in division, extending the possibilities of detecting anomalies not otherwise visible (particularly when only numerical chromosomal changes are to be ascertained). FISH is gaining increasing popularity, particularly because in addition to being an easy procedure for the detection of specific sequences in interphase or metaphase cells, it can also be applied to fixed and paraffin embedded tissues *(6–8)*. However, FISH approaches also suffer from the shortcoming of the investigator having to know a priori which probes are to be used in each case being examined. The use of FISH based on painting and cosmid probes also requires knowledge regarding the exact anomalies to be ascertained. Detection sensitivity for FISH and other techniques is shown in **Table 1**.

Table 1
Comparison of FISH with Other Assays

Technique	Marker	Detection limits
Routine pathology	Cellular morphology	10^{-1}–10^{-2}
Cytogenetics	Chromosome morphology	10^{-1}–10^{-2}
FISH	Chromosome structure	10^{-2}
Gene rearrangement	DNA configuration	10^{-2}–10^{-3}
FACS analysis	Antigen profile	10^{-3}
Clonogenic culture	In vitro growth	10^{-5}
PCR	DNA/RNA structure	10^{-5}

FACS, fluorescence activated cell sorting; PCR, polymerase chain reaction.

1.3. Clinical Applications of FISH in Hematologic Malignancies

During the last decade there has been an exponential increase in the application of FISH techniques to various facets of human genetics ***(7–11)***. The rapid advances in the human genome effort, and the continuing elucidation of the genetic pathways of human diseases, have yielded readily available nucleic acid reagents required for the clinical application of FISH technology. FISH has been widely used to study the genetic events underlying hematopoietic disorders and to classify these disorders in a meaningful way, as well as to monitor the response to various therapeutic interventions (*see* **Table 2**). Both numerical and structural chromosome abnormalities are amenable to FISH analysis. A brief overview of examples of application of FISH in the study of hematologic disorders is given here.

1.3.1. Acute Lymphoblastic Leukemia (ALL)

Hyperdiploidy is found in 16–23% of adults and in up to 40% of children with ALL. The favorable prognosis associated with high hyperdiploidy (51–68 chromosomes) in children and adults with ALL is well established. FISH has been reported to potentially detect these cases with aneuploidy. Utilizing probes for 10 chromosomes (X, 4, 6, 8, 10, 14, 16, 18, 20, and 21), in particular combinations and in a stepwise manner, Moorman et al. ***(9)*** detected hyperdiploidy with FISH techniques in 94% of such cases and gave an accurate prediction of ploidy subgroups in 96% of these cases in a model population of 252 ALL cases. Our observations are also compatible with these findings. Therefore, these approaches may identify missing or hidden hyperdiploid cases among cases that have not been successfully analyzed cytogenetically.

Table 2
FISH Applications in Hematologic Disorders

Detection of diagnostic numerical and structural anomalies
Marker chromosome identification
Detection of gene amplification
Analysis of terminally differentiated or nondividing cells
Analysis of fixed or nonviable cells
Monitoring course of disease
Monitoring effects of therapy
Identification of the origin of a graft postallogenic bone marrow transplantation

1.3.2. Acute and Chronic Myeloid Malignancies

FISH, utilizing centromeric and unique sequence probes, has cogent and practical application in myeloid malignancies, including acute nonlymphocytic leukemia (ANLL), chronic myelocytic leukemia (CML), myeloproliferative disorders (MPD), and myelodysplastic syndromes (MDS), where it can be used to characterize these disorders, e.g., monosomy 7 (–7) and trisomy 8 (+8) in MDS, +8 and +9 in MPD, t(9;22) in CML, and t(15;17) in ANLL ***(11,12)***.

1. Anomalies of 11q23: Reciprocal translocations involving chromosome 11 at band q23 have been observed in both ALL and ANLL. The incidence of 11q23 abnormalities has been estimated to be approximately 5% in adult and childhood ALL and 75% or greater in infant leukemias. By FISH it was shown that most 11q23 rearrangements involve the same breakpoint cluster region of the MLL gene, although heterogeneity in the breakpoints in some of the rare rearrangements exists ***(13)***.
2. FISH in combination with morphology (MGG/FISH) was also used to detect minimal residual disease (MRD) in complete remission (CR) in leukemia patients with numerical chromosome aberrations at diagnosis. The results indicate that MGG/FISH may be a clinically useful method to detect MRD in acute leukemia and predict relapse, particularly when repeat studies are performed during CR ***(14)***.

1.3.3. Chronic Lymphocytic Leukemia (CLL)

Chromosomal abnormalities have been described in about 50% of CLL patients using conventional cytogenetic methodologies. The most common abnormalities are trisomy 12 (+12) in 10–18% of cases and structural abnormalities of 13q14 in 10–28% of cases. However, accurate and successful cytogenetic analysis of specimens has been hindered by the low in vitro mitotic activity of the critical cell population and culture failure in up to 40% of the cases of CLL studied. Analysis of interphase cells provides a sensitive tool for

the detection of numerical cytogenetic abnormalities in poorly dividing cells. With FISH techniques, trisomy 12 has been reported in up to 63% of CLL cases ***(15)***.

Molecular studies, including FISH, have also demonstrated allelic deletion of the RB1 gene in 21–30% of CLL cases, and of the D13S25 marker, one megabase (Mb) telomeric to RB1, in 24–60% of the cases. Recently, FISH studies have provided further evidence for the existence of a new tumor suppressor locus in B-cell CLL located at 13q12.3 ***(16)***. BRCA2, located within the minimal deletion consensus, is a candidate for the gene. Interestingly, in most conventional chromosome banding studies of B-CLL, 11q deletions have not been identified as a frequent change. However, with FISH using the yeast artificial chromosome (YAC) clone 755b11 from the chromosome region 11q22.3-23.1, 11q deletions (20%) were found to be the second most frequent chromosome aberration following 13q14 deletions ***(17)***.

1.3.4. Lymphoma

The most common characteristic chromosome abnormalities in B-cell non-Hodgkin's lymphoma (NHL) are translocations involving 14q32, such as t(8;14)(q24;q32) in Burkitt's lymphoma, t(14;18)(q32;q21) in follicular NHL, t(11;14)(q13;q32) in intermediate lymphocytic lymphoma/mantle-cell lymphoma, and t(3;14)(q27;q32) in diffuse lymphomas with large-cell components. However, cytogenetic investigations are not always successful in lymphoma, due to poor or lack of metaphase spreads and suboptimal chromosomal morphology. Recently, it was reported that a set of probes for interphase FISH analysis has been successfully established for the detection of tumor-specific rearrangements of the immunoglobulin heavy-chain (IgH) gene in B-cell malignancies ***(18)***. The results indicate that interphase FISH with IgH gene probes may be a rapid and reliable method to identify lymphoma-related gene rearrangements. As mentioned before, 50–75% of mantle-cell lymphomas (MCL) are associated with the t(11;14)(q13;q32). Using Southern blot analysis, a BCL1 breakpoint can be detected in about 50% of MCL cases. Utilizing FISH with two probe sets of differently labeled cosmids, symmetrically localized at either side of the major translation cluster of BCL1, it was reported that this FISH approach can be used to distinguish the t(11;14) from other 11q13 rearrangements in hematologic malignancies ***(19)***. Following the same strategy, the t(2;5)(p23;q35), that occurs in 25–30% of anaplastic large-cell lymphoma, was also reported to be successfully detected by interphase FISH ***(20)***. Furthermore, numerical chromosomal abnormalities in NHL were also investigated with interphase FISH. One study indicated that trisomy 12 (+12) was detected in 33% of the patients with follicular lymphoma, polysomy 12 in 37% of patients with diffuse large-cell lymphoma, monosomy 18 in 43% of

cases with CLL, and 28% of those with small-cell lymphocytic lymphoma, trisomy, or tetrasomy 17 in 27% of NHL patients, and X-chromosome aneuploidy in patients with NHL ***(21)***.

2. Materials

2.1. Specimens

Due to the high stability of DNA, FISH can be performed on most specimens, ranging from blood and bone marrow smears, buccal smears, cytospins, and touch print preparations to archival pathology specimens and epithelial cells in bladder washings and urine. Logically, any nucleus can be evaluated with FISH methods as long as the DNA in the cell is not degraded (*see* **Note 1**).

For hematological disorders, bone marrow (BM) and peripheral blood (PB) are usually the specimens submitted for FISH analysis. Often these samples are first processed for chromosomal analysis and FISH is performed on the remaining fixed-cell pellet in cases of unsuccessful cytogenetics, to optimally interpret the observed abnormality or as a monitoring tool during treatment. BM is an ideal tissue for the observation of the in vivo chromosomal situation. Unstimulated blood cells are examined in order to observe the leukemic cells spontaneously dividing in the PB. Stimulated PB cells are used to examine T-cell or B-cell types that may be involved in specific lymphocytic diseases.

BM and PB smears also can be used for rapid FISH analysis. Lysis of red blood cells and fixation of cells can be accomplished after the smear is made. Generally, this kind of preparation can be employed with any DNA probe.

2.2. DNA Probes

Three major categories of DNA sequences are used for probes in FISH analysis.

2.2.1. Centromere-Specific Alpha Satellite DNA Sequence Probes

The most popular type of probe consists of the chromosome centromere-specific alpha satellite DNA sequences which have a 171-bp DNA monomer, and are tandem repetitive and polymorphic, and do not code for a gene product ***(22)***. The alpha satellite DNA is almost identical in all human chromosomes except for 2–3% of the DNA, which is variable to the degree that centromeres of each individual chromosome can be distinguished and probes to these chromosomes can be generated ***(23)***. Other repetitive DNA sequence probes include those produced from the beta satellite DNA, which consists of a 68-bp monomer arranged in the same fashion as the alpha satellite DNA and is located at the tip of each acrocentric chromosome ***(24)***, as well as the classical satellite I DNA, which is an AATGG repeat found on chromosomes 1, 9, 15,

16, and Y ***(25)***. The major use of these satellite DNA probes is in the rapid enumeration of chromosomal monosomies or trisomies (*see* **Fig. 2**). Because the targets are large and repeated many times, these probes generate large signals.

2.2.2. Sequence-Specific Sequence Probes

The detection of unique single-copy genes (*see* **Fig. 3**) is accomplished by the use of sequence-specific probes. Levels of detection range from sequences as small as 1 kb up to as large as 300 kb ***(26)***. The various FISH unique-sequence probes are usually employed to detect microdeletion syndromes and rearrangements of oncogenes. Subtelomeric probes are produced from unique sequences in close proximity to the ends of chromosomes and are often used for the analysis of cryptic translocations.

2.2.3. Whole Chromosome and Arm-Specific Sequence Probes

Whole chromosome probes (WCP) and chromosome arm-specific probes consist of numerous unique and repetitive sequences from an entire or a partial chromosome. They can be derived from somatic cell hybrids; single flow sorted chromosomes, or microdissection of specific chromosomes with PCR amplification of the dissected DNA ***(27,28)***. These probes are primarily designed for application on metaphase chromosomes in analysis of markers and complex chromosomal rearrangements.

2.3. Probe Labeling

In situ hybridization was successfully performed in the past with the use of light microscopic detection methods utilizing horseradish peroxidase and other immunocytochemical reagents. However, except for the alpha satellite probes, unique sequence *in situ* hybridization probes cannot be easily resolved using a light microscope. FISH probes are more readily visualized with fluorescence microscopy.

Direct and indirect procedures are the two types of commonly used nonradioactive hybridization methods. Incorporation of nonisotopic reporter molecules into probes is achieved enzymatically or chemically ***(29)***. In the direct procedure, probe nucleotides are directly labeled with fluorochromes. The bound probe and target can be visualized directly with fluorescence microscopy. Incorporation of fluorochromes into probes can be accomplished with the use of polymerase enzymes and labeled nucleoside triphosphates ***(30)***. In the indirect method, DNA probes are tagged with a hapten, the most commonly used being biotin or digoxigenin. Biotin binds to avidin or streptavidin with high affinity and is used for the detection of biotin-labeled probes. Antibodies to digoxigenin are used for the detection of digoxigenin-labeled probes.

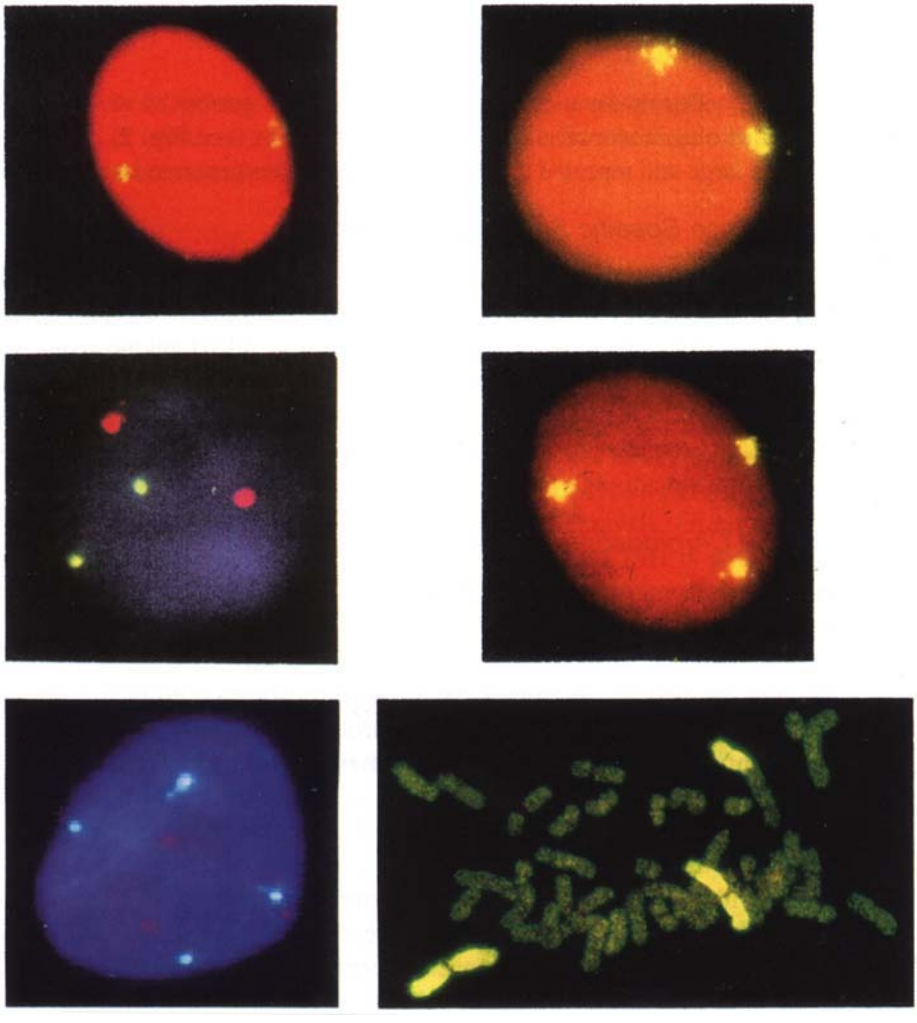

Fig. 2. In this plate are shown some representative results obtained with FISH. (upper left) Two signals obtained with a centromeric probe for chromosome 7 in a normal interphase cell. (upper right) Two signals obtained with a centromeric probe for chromosome 8 in a normal interphase cell. (middle left) Signals obtained when two differently labeled probes (chromosomes 7 and 9) were applied to a normal interphase cell. The red signals are those of chromosome 7 and the yellow-green for chromosome 9. (middle right) A leukemic interphase marrow cell showing three signals for the centromeric probe of chromosome 8. This finding indicates trisomy 8 (+8) to be present. (lower left) An interphase cell from a case with myelodysplastic syndrome showing three red signals for a centromeric probe for chromosome 8 (trisomy 8) and four blue signals for the probe for chromosome 10 (tetrasomy 10). (lower right) FISH using chromosome painting for chromosome 1 in a bone marrow metaphase. Two normal chromosomes 1 are present, as well as a derivative chromosome (upper right of metaphase) containing one of the arms of a chromosome 1.

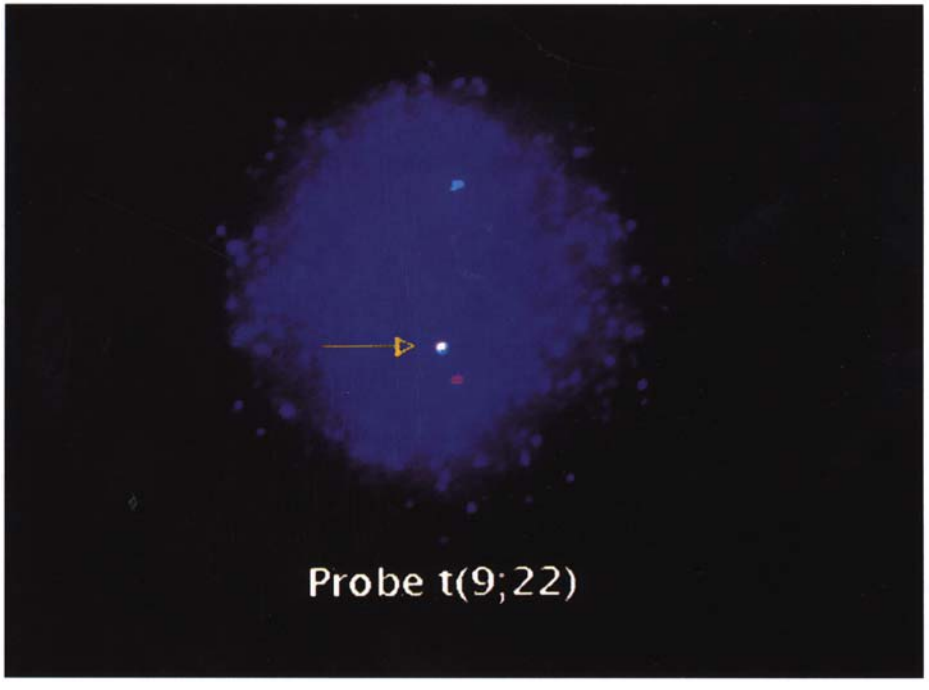

Fig. 3. Demonstration with FISH of the fusion product of the Ph translocation, t(9;22)(q34;q11) using a cosmid probe (arrow) for the translocation product.

Where a hapten is used as the reporter molecule, labeling methods include nick translation ***(31)***, random priming ***(32)***, in vitro transcription ***(33)***, and PCR amplification ***(34)***.

Multicolor labeling and detection have also gained popularity due to the flexibility of using labeling reagents. Two or three distinguishable colors can be visualized concurrently to study various targets of interest.

Counterstains help visualize the surrounding DNA or background nuclear material, the commonly used ones being propidium iodide (PI) and diamidino-2-phenylindole (DAPI). Both are DNA intercalators and fluorescent under similar wavelengths as are other fluorochromes, such as fluorescein, Texas Red, rhodamine, spectrum orange, and spectrum green. In general, when using a red fluorochrome, such as Texas Red or rhodamine or a dual-labeling study, such as a red and green/yellow dye analysis, the blue DAPI counterstain is the ideal choice; when using a green fluorochrome, such as fluorescein or spectrum green, PI counterstain is the best. An antibleaching chemical is commonly used to preserve the signal during storage and photography. Fading of fluorochromes on excitation is a photochemical process. Mounting media containing

diphenylene diamine or other agents act as radical scavengers and antioxidants that alleviate the quenching without altering the experimental results.

2.4. Reagents

2.4.1. Prehybridization and Hybridization

1. Herring sperm DNA (0.5 mg/mL).
2. RNase (1.0 mg/mL).
3. 70, 85, and 100% ice-cold ethanol.
4. Denaturing solution: 70% formamide, 2X standard saline citrate (SSC) (pH 7.0).
5. Hybridization master mix (MM 2.1): 55% formamide, 10% dextran sulfate, 1X SSC.
6. Rubber cement, prepared slides, and coverslips.

2.4.2 Posthybridization Washes and Signal Detection

1. Wash solutions (stringency-dependent on type of probes): 50% formamide, 2X SSC (pH 7.0).
2. PN buffer: 0.1 *M* NaPO4 (pH 8.0), 0.1% Nonidet P-40 (NP-40).
3. PNM buffer: NaAzide in PN (0.2 mg/mL).
4. 2X SSC (pH 7.0).
5. 2X SSC (pH 7.0), 0.1% NP-40.
6. 0.1X SSC (pH 7.0).
7. Antidigoxigenin-fluorescein isothiocyanate (FITC) (20 µg/mL in PNM or Oncor), or-rhodamine (Oncor).
8. Rabbit anti-sheep antibody I (Oncor).
9. Anti-rabbit antibody II-FITC (Oncor) or rhodamine (Oncor).
10. Avidin-FITC (5 µg/mL in PNM or Oncor).
11. Biotinylated anti-avidin (5 µg/mL in PNM or Oncor).
12. Antifade solution: P-phenylenediamine dihydrochloride in PBS (10 mg/mL).
13. PI (1 µg/mL).
14. DAPI (1 µg/mL).
15. Mounting medium: antifade solution, counterstain, i.e., PI or DAPI (self-made or Oncor or Vysis).

2.5. Instruments

1. Centrifuge
2. Thermometers
3. Timers
4. Pipetman
5. Waterbaths
6. Incubator
7. Slide warmer
8. Refrigerator, freezer
9. pH meter
10. Balance
11. Forceps

3. Methods

3.1. Slide Preparation

3.1.1. Fixed Cell Pellet

Metaphase or interphase cell slides are prepared from fixed BM or PB cell suspensions by conventional cytogenetic techniques in such a way that most of the cytoplasm is not visible around the metaphases and nuclei. Slides are air-dried for 10 min to overnight. Baking the slides in an oven is not recommended. Until FISH can be performed, the slides are stored in 70% ethanol at 4°C for a minimum of 2 h to a maximum of 2 wk. Best results are achieved when slides are used within the first 2 wk.

Fresh slides can be used without pretreatment. Slides older than a week should be pretreated as follows:

1. Incubate in RNase A (1.0 mg/mL) for 1/2–1 h at 37°C.
2. If a heavy cytoplasm is present, treat the slides further in 100% acetic acid for 1/2 h.
3. Dehydrate the slides in 70, 85, and 100% ethanol series for 1 min each at room temperature. Air-dry.

3.1.2. BM and PB Smears

1. Allow freshly made smear slides to air-dry for 10–30 min at room temperature.
2. Fix the slides for 5 min in 100% methanol. Air-dry.
3. Apply 50 µL of RNase A (1.0 mg/mL) solution onto each slide, adding a 25 × 25mm glass coverslip and incubate it at 37°C for 30 min.
4. Rinse the slides with distilled water.
5. Place the slides in 2X SSC at 37°C for 30 min.
6. Place the slides in 70, 85, and 100% ethanol series at room temperature for 2 min each.
7. Air-dry. Subsequently perform a FISH analysis.

3.1.3. Giemsa-Pretreated Slides

1. Fix slides for 15–20 min in 100% methanol two times.
2. Place the slides in 70, 85, and 100% ethanol series at room temperature for 1 min each.
3. Place the slides in 3:1 methanol:glacial acetic acid for 10 min. Air-dry.
4. Wash the slides in 3.7% formaldehyde for 10 min and phosphate-buffered saline (PBS) for 5 min two times.
5. Place the slides in 70, 85, and 100% ethanol series at room temperature for 1 min each. Air-dry. Subsequently perform a FISH analysis.

3.2. FISH Procedures

Probes available commercially are accompanied by the manufacturer's suggestions on probe preparation and hybridization. Detection kits are also available from various manufacturers. Presented here are the procedures we use in our laboratory, which were established on the basis of the literature ***(3,35)*** and some of the commercial manufacturers' guidelines (Vysis, Oncor) with modifications. The procedures for directly labeled probes are primarily based on Vysis procedure guidelines.

3.2.1 Repetitive Sequences Probes

3.2.1.1. Indirectly (Digoxigenin or Biotin)-Labeled Probes

1. Denaturation and Hybridization:
   a. Use a previously refrigerated Coplin jar containing denaturing solution and place it in a waterbath. Turn on the waterbath and bring the temperature up to 70–72°C inside the Coplin jar. Before hybridization place a clean thermometer into the Coplin jar to check the exact temperature of the hybridization solution. The denaturing temperature of 70°C is critical, and each slide that is placed into the solution will drop the temperature one degree. Denature no more than two slides at a time. If the waterbath has been turned on and is already up to the temperature, place denaturing solution in a 37°C waterbath for 10 min, then in a 65°C waterbath for 10 min, and finally in a 72°C waterbath.
   b. Turn on a warming tray to 40°C and wash off the surface with 70% ethanol.
   c. Use a cold ethanol series (70%, 85%, 100%) previously kept in a freezer. Do this just before starting to denature slides.
   d. Place slides in the denaturing solution for 2 min.
   e. Dehydrate the slides in the cold ethanol series (70%, 85%, 100%) for 2 min each, with some agitation.
   f. Air-dry slides.
   g. Label probe vials with the probe to be made.
   h. Prepare probe mixtures. For each 22 × 22 mm coverslip, use 10 µL of probe mixture. A 22 × 50 mm coverslip requires 20 µL of probe mixture.
      i. 7 µL MM 2.1
      ii. 1 µL carrier DNA (0.5 mg/mL)—herring sperm
      iii. 2 µL probe DNA (0.5 mg/mL)
   i. Vortex probe vials and microfuge for a short time to mix and concentrate probe mixtures in the bottom of vials.
   j. Use a microtube floating rack, float probe mixtures in a 72°C waterbath for 5 min.
   k. Immediately chill probe mixtures in a freezer for approximately 2 min.
   l. Vortex and microfuge to collect all droplets.
   m. Place the air-dried slides and probe mixtures on a warming tray. Bring slides, probe mixtures, coverslips, pipet tips and moist chambers to approximately 37–40°C.

n. Pipet probe mixture onto each slide and add a coverslip, trying to avoid the formation of air bubbles. If there are air bubbles under a coverslip, press the coverslip with forceps and work bubbles to the sides of the coverslip. Once the probe is on a slide, the temperature should never be allowed to drop below 37°C, as this can cause nonspecific binding which will not wash off (*see* **Note 2**).
o. Use a 10-cm^3 syringe to seal edges of coverslips with rubber cement.
p. Place the slides in warm, moist chambers and place these into a 37°C incubator overnight.

2. Post-hybridization wash:
   a. Turn on a warming tray to 40°C.
   b. Remove three wash jars from refrigerator and place them in order in a cool waterbath. (Placing cold jars into a hot bath may cause the jars to shatter.) Turn on the waterbath to an appropriate washing temperature. Allow approximately 30 min for the waterbath to equilibrate. The recommended temperature is good for two slides; temperature has to be adjusted to accommodate more slides (0.5°C higher per slide added).
   c. Remove moist chambers from the incubator. Place chambers on the warming tray (40°C).
   d. Peel off rubber cement with forceps. Place slides in the first wash solution, let sit for a minute or so, and then remove coverslips with forceps. Coverslips should just slide off without difficulty. If tension persists, let them sit in the wash solution a little longer. DO NOT pull up coverslips, as this will damage the cells. Once the coverslip is off, agitate slide(s) and incubate for 2 min.
   e. Wash the slides in washes 2 and 3, respectively, for 2 min each, with agitation. Always use jars in the same order.
   f. Place the slides in 2X SSC (pH 7.0) at room temperature for 2 min.
   g. Follow with 2 min (minimum time) wash in PN buffer at room temperature. Slide(s) can remain in PN buffer for hours at room temperature or even overnight at 4°C before proceeding to the next step.
3. Detection (for digoxigenin-labeled probes):
   a. Remove the slides from PN buffer and blot excess fluid from the edge. Do not allow the slide surface to dry; this will cause nonspecific binding of the detection reagent and high background fluorescence (*see* **Note 3**).
   b. Apply 30 µl of fluorescein-labeled anti-digoxigenin or rhodamine-labeled anti-digoxigenin to each slide and place a plastic coverslip over the solution. Incubate the slides at 37°C for 5 min in prewarmed humidified chambers.
   c. Take the humidified chambers out of the incubator and place them on a warming tray (37°C). Dip the slides in PN buffer to remove coverslips.
   d. Wash the slides three times for 2 min each in 40 ml of PN buffer at room temperature. These washes remove excess detection compounds.
4. Digoxigenin Amplification
   a. Remove the slides from PN buffer and blot excess fluid from the edge. Do not allow slide surface to dry.

b. Apply 20 µL of rabbit anti-sheep antibody I to each slide and place a plastic coverslip over the solution. Incubate the slides at 37°C for 15 min in a prewarmed humidified chamber.
c. Dip the slides in PN buffer to remove coverslips. Wash the slides three times for 2 min each in 40 mL of PN buffer at room temperature.
d. Apply 20 µL of fluorescein or rhodamine-labeled anti-rabbit antibody II to each slide and place a plastic coverslip over the solution. Incubate the slides at 37°C for 15 min in a prewarmed humidified chamber.
e. Dip the slides in PN buffer to remove coverslips. Wash the slides three times for 2 min each in 40 mL of PN buffer at room temperature.
f. Counterstain with 10 µL mounting medium. Place a glass coverslip on each slide, remove any bubbles, and blot excess PI or DAPI by placing the slide between two pieces of bibulous paper and pressing on the slide. View with a fluorescent microscope (*see* **Note 4**).
g. Keep slides in a light-tight box until they are scored. They can be kept at 4°C for 7–10 d.

5. Detection (for biotin-labeled probe):
   a. Take the slides out of PN buffer and add 20 µL of avidin to each slide, place a plastic coverslip over the solution, place the slides in moist chambers, and incubate them for 5 min in an incubator at 37°C.
   b. Wash the slides three times in fresh PN buffer at room temperature for 2 min each with agitation. Coplin jars must be wrapped in foil. Signal will decrease with exposure to light. With some of the more repeated probes the signal may be visible at this point, however, we usually proceed with one round of amplification.
6. Amplification for biotin-labeled probe:
   a. Apply 20 µL of anti-avidin to each slide. Place a plastic coverslip over the solution. Place the slides in moist chambers and incubate them at 37°C for 5 min.
   b. Wash the slides three times in PN buffer at room temperature for 2 min each with agitation.
   c. Apply 20 µL of avidin to each slide, place a plastic coverslip over the solution. Place the slides in moist chambers and incubate them at 37°C for 5 min.
   d. Wash the slides three times in fresh PN buffer at room temperature for 2 min each with agitation. (For very weak probes, **steps a–d** can be repeated to obtain a second round of amplification and therefore a brighter signal; however the background will also be increased, *see* **Note 5.**)
   e. Drain excess fluid from the slides but do not allow the slides to dry. Pipet 10 µL of PI/antifade or 10 µL of DAPI for each 22 × 22 mm coverslip onto the slide (20 µL for a 22 × 50 mm coverslip).

3.2.1.2. Directly-Labeled Probes

1. Probe preparation:
   a. At room temperature, mix 7 µL of CEP hybridization buffer (Vysis), 1 µL of directly labeled CEP DNA probe and 2 µL sterile deionized water in a

microcentrifuge tube. For dual color, mix 7 µL of CEP hybridization buffer, 1 µL spectrum orange DNA probe, 1 µL spectrum green DNA probe, and 1 µL sterile deionized water.

b. Centrifuge 1–3 seconds in a microcentrifuge.

2. Denaturation and hybridization:
   a. Remove the Coplin jar containing denaturing solution from the refrigerator and place it in a 70–75°C waterbath, which has been turned off. Turn on the waterbath and bring temperature to 70–75°C. (Placing a cold Coplin jar in hot waterbath may cause the jar to shatter.)
   b. Turn on a warming tray to 45°C.
   c. Denature DNA probe mixture for 5 min in a 70–75°C waterbath.
   d. Denature slides in the denaturing solution for 5 min.
   e. Wash the slides 1 minute each in cold ethanol series (70%, 85%, and 100%).
   f. Air-dry the slides.
   g. Place slide pipet tips, the probe mixture, and coverslips on the slide warmer (45°C).
   h. Pipet 10 µL of probe mixture onto each slide, adding a coverslip, and seal edges with rubber cement.
   i. Place the slides in humidified chambers and incubate them for 16–24 h (overnight) in a 37°C incubator.
3. Post-hybridization wash and detection:
   a. Turn on a slide warmer to 45°C and place the humidified chambers on the slide warmer.
   b. Place three wash solutions in a waterbath and bring the temperature up to 45°C.
   c. Remove rubber cement and coverslips.
   d. Wash the slides three times for 10 min each in 45°C wash solutions, keeping solutions in correct order. No more than two slides should be processed per wash procedure.
   e. Wash the slides for 10 min in 2X SSC at 45°C.
   f. Wash the slides for 5 min in 2X SSC/0.1% NP-40 (40 µL NP-40 in 40 mL 2X SSC) at 45°C.
   g. Allow the slides to air-dry in darkness.
   h. Apply 10 µL mounting medium (for two-color hybridization, DAPI counterstain works best) and a coverslip to each slide.
   i. Store the slides in a light-tight box until they are ready to be scored. They can be kept at –20°C.

3.2.2. Unique Sequences Probes

3.2.2.1. Indirectly (Digoxigenin or Biotin)-Labeled Probes

1. Slide pretreatment: Place slides in 2X SSC (pH 7.0) at 37°C for 30 min. Dehydrate the slides at room temperature in 70, 85, and 100% ethanol for 2 min each. Air-dry.

2. Slide denaturation: Denature the slides in denaturing solution at 70°C for 2 min; dehydrate the slides in cold 70, 85, and 100% ethanol series for 2 min each and air-dry.
3. Probe preparation and hybridization:
   a. Prewarm probe mixture (Oncor) at 37°C for 5 min. DO NOT HEAT DENATURED PROBES.
   b. Vortex the probe mixture and microfuge before pipetting.
   c. Prewarm the slides in a humidified chamber at 37°C.
   d. Apply 20 µL of probe mixture per 22 × 50 mm coverslip or 10 µL of probe mixture per 22 × 22 mm coverslip to each slide. Apply glass coverslips and seal with rubber cement. Incubate the slides for 16–24 h at 37°C in humidified chambers.
4. Posthybridization wash:
   a. Use the series of three washes (50% formamide/2X SSC pH 7.0) for 5 min each at 43°C.
   b. Place the slides in 2X SSC, pH 7.0 at 37°C for 8 min.
   c. Transfer the slides to PN buffer for 2 min.
5. Detection: See the indirectly labeled repetitive sequences probe procedure in **Subheading 3.2.1.1.** for the digoxigenin-labeled or biotin-labeled detection and amplification.

3.2.2.2. Directly Labeled Probes

1. Slide pretreatment: The same procedure as for indirectly labeled unique sequences probes.
2. Probe preparation:
   a. At room temperature, mix 7 µL of large-scale integration (LSI) hybridization buffer (Vysis), 1 µL of directly labeled cosmid DNA probe and 2 µL sterile deionized water in a microcentrifuge tube.
   b. Centrifuge 1–3 s in a microcentrifuge.
3. Denaturation and hybridization:
   a. Denature DNA probe mixture for 5 minutes in a 70–75°C waterbath.
   b. Denature slides in denaturing solution at 70–75°C for 5 min.
   c. Wash the slides 1 min each in cold 70, 85, and 100% ethanol series.
   d. Air-dry the slides.
   e. Place slide pipet tips, the probe mixture, and coverslips on the slidewarmer (45°C).
   f. Pipet 10 µL of probe mixture onto each slide, adding a coverslip, and seal edges with rubber cement.
   g. Place the slides in humidified chambers and incubate them for 16–24 h (overnight) in a 37°C incubator.
4. Post-hybridization wash and detection:
   a. Wash the slides three times for 10 min each in 45°C wash solutions, keeping solutions in correct order. No more than two slides per wash procedure.
   b. Wash the slides for 10 min in 2X SSC at 45°C.

c. Wash the slides for 5 min in 2X SSC/0.1% NP-40 (40 µL NP-40 in 40 mL 2X SSC) at 45°C.
d. Allow the slides to air-dry in darkness.
e. Apply 10 µL mounting medium (for two-color hybridization, DAPI counterstain works best) and a coverslip to each slide.
f. Store the slides in a light-tight box until they are ready to be scored. They can be kept at –20°C.

3.2.3. Whole Chromosome Painting Probes

3.2.3.1. Indirectly (Digoxigenin or Biotin)-Labeled Probes

1. Probe preparation:
   a. Prewarm probe mixture (Oncor) at 37°C for 5 min.
   b. Aliquot 10 µL of the probe mixture into a microcentrifuge tube.
   c. Denature the probe mixture at 70°C for 10 min.
   d. Incubate the probe mixture at 37°C for 2 h to preanneal.
2. Denaturation and hybridization:
   a. Denature slides in denaturing solution at 70°C for 2 min.
   b. Dehydrate the slides in cold ethanol series (70, 85, 100%) for 2 min each, with some agitation.
   c. Air-dry slides.
   d. Pipet 10 µL of probe mixture onto each slide, adding a coverslip, and seal edges with rubber cement.
   e. Place the slides in humidified chambers and incubate for 16–24 h (overnight) in a 37°C incubator.
3. Posthybridization wash:
   a. Wash the slides for 5 min in each of three washes (43°C).
   b. Place the slides in 0.1X SSC, pH 7.0 at 60°C for 8 min.
   c. Place the slides in PN buffer for 2 min.
4. Detection: See the indirectly labeled repetitive sequences probe procedure in **Subheading 3.2.1.1.** for digoxigenin-labeled or biotin-labeled detection and amplification.

3.2.3.2. Directly Labeled Probes

1. Probe preparation:
   a. Allow whole chromosome painting (WCP) hybridization buffer (Vysis) to warm to room temperature so that its viscosity decreases to the point that it may be accurately pipetted.
   b. In a microcentrifuge tube add 7 µL WCP hybridization buffer, 1 µl WCP DNA probe, and 2 µl deionized water. This quantity of probe mixture is sufficient to cover one 22 mm × 22 mm hybridization zone.
   c. To screen samples for two WCP probes simultaneously, prepare probe mixture as follows: 7 µL WCP hybridization buffer, 1 µL spectrum orange WCP DNA probe, 1 µL spectrum green WCP DNA probe, and 1 µL deionized water.

d. DAPI counterstain must be used for visualization, as PI counterstain will fluoresce in the same region of the spectrum as the spectrum orange fluorophore.
e. Denature the probe mixture for 5 min in a 73°C waterbath.
f. Cool the probe mixture in a freezer for 1–2 min.

2. Denaturation and hybridization:
   a. Immerse slides in 70–73°C denaturing solution for 5 min to denature the target DNA. To maintain the temperature of the denaturing solution, place no more than two slides in denaturing solution at one time. Longer or shorter denaturation time, for example 2–10 min, may be necessary for some specimens.
   b. Dehydrate the slides at room temperature in 70, 85, and 100% ethanol wash solutions for 2 min each.
   c. Place the slides on a 45°C slide warmer.
   d. Leave the slides on the slide warmer and apply the aliquot of the probe mixture to the target area of each slide. Place a prewarmed glass coverslip over the probe mixture and seal the edges with rubber cement.
   e. Place the slides in preheated humidified chambers.
   f. Place the chambers in a 37°C incubator. Allow hybridization to proceed for at least 4 h or, preferably, overnight.
3. Posthybridization wash and detection:
   a. Wash the slides for 10 min in each of three washes (44°C).
   b. Wash the slides in a jar containing 2X SSC, pH 7.0, preheated to 44°C for 10 min.
   c. Wash the slides in a jar containing 2X SSC/0.1% NP-40 preheated to 44°C for 5 min with agitation.
   d. Air-dry the slides in darkness.
   e. Apply 10 µL of mounting medium to the target area of each slide. Place a coverslip over the counterstain.
   f. Place the slides in black boxes: the slides are now light sensitive and signals will fade if exposed to light.

3.2.4. Visualization—Image Recording

Blue, green, and UV filter sets (e.g., Zeiss filter sets: 01, 09, 15; Nikon filter sets: G-20, B-12, UV-10) with a good fluorescence microscope, such as Zeiss Axioplan, Zeiss Axiophot, and Nikon Microphot FX, are necessary elements for the visualization of FISH results. For DAPI/fluorescein, Ektachrome 160 tungsten film works well. Kodacolor 400 and Fujichrome 400 are the better choices for photographing the red and yellow of PI/fluorescein.

Digital imaging systems also are now widely used in FISH analysis. These apparatuses consist of a combination of microscope, camera, and computer with advanced software, allowing the recording of images electronically by using video or low-light cameras. These devices are particularly useful for detailed FISH analysis of small signals, from phage and cosmid probes, or YACs crossing translocation breakpoints (*see* **Note 6**).

3.3. Regulations, Controls, and Analysis

The American College of Medical Genetics (ACMG) has developed some policies and quality assurance guidelines for the clinical application of FISH *(36)*. The Food and Drug Administration (FDA) has also approved several DNA FISH probes for clinical use. However, in general, FISH still is considered an investigational technique with conventional cytogenetic results ultimately serving as the primary diagnostic test.

Probe validation and controls for probes and types of specimens should be established when performing FISH analysis *(36)*. Probe validation assures that probes employed will produce the most successful hybridization with the highest analytical specificity and sensitivity. Controls will provide essential information about the success of an experiment and the criteria to evaluate the results of FISH studies. Finally, clinical validation of FISH procedures and results is important, because it will afford laboratory workers the opportunity to gain appropriate experience in the performance of the test system.

Because there are numerous sources of variation in FISH data between laboratories, e.g., differences in preparing samples, probes employed, FISH procedures, experience, and subjective counting criteria between observers, it is important that analysis criteria for interphase and metaphase FISH should be established for each laboratory performing FISH utilizing various probes on different specimens. A case in point are the criteria for scoring interphase FISH, including at least two technologists scoring the same case, examination of a large number of cells, avoiding damaged and overlapping nuclei, as well as areas of the slide where hybridization is absent or suboptimal, focusing up and down on each nucleus and so on. In addition, reporting criteria of FISH results are also important, which should include the probes used, the source and identification of the probes used, the number of test and control cells scored and detailed, hybridization results, limitations of the assay, and following ISCN 1995 *(37)* for FISH nomenclature.

4. Notes

1. No signal with a probe that has performed well previously: This situation may be related to the probe, which has degraded because of improper handling and/or shipping. It is important that FISH probes be stored at –20°C and handled with gloves and autoclaved pipet tips. A change in sample type and sample degradation may also influence probe signal intensity.
2. Cross-hybridization (nonspecific fluorescent signals): Because exact pairing of DNA sequences is achieved and maintained under certain conditions, more stringent reaction conditions may be necessary to reduce crosshybridization, which can be accomplished by increasing the temperature of hybridization and rinses,

increasing formamide concentration or decreasing the concentration of salts (e.g., SSC). For blocking nonspecific hybridization signals when painting probes are being used, Cot-1 fraction of total human genomic DNA is often successful.

3. Nonspecific background: Components of blocking agents are often responsible for these problems. Changing blocking components or detection systems or preparing fresh solutions are helpful in solving these problems.
4. Suboptimal signal intensity: Common problems may be related to the microscope, including either the bulb alignment or the filter sets. In addition, there are several strategies to maximize signal intensity.
   a. Diluting the counterstain with antifade until it is just bright enough to scan with a low-power objective (e.g., PI: 0.3 µg/mL and DAPI: 0.05 µg/mL).
   b. The use of amplification as described in the **Subheading 3.2.1.1.**
   c. Repeating the hybridization using a lower-stringency wash.
5. Cytoplasmic background: Increased cytoplasmic background can reduce probe nuclear penetration and cause suboptimal hybridization. These conditions can be improved when the slide preparations are pretreated with proteinase-K (e.g., 0.6 mg/ml in 20 mmol/L Tris HCl, 2 mmol/L CaCl2, pH 7.5 for 1–5 min at 40°–42°C) and/or RNase.
6. Other powerful cytogenetic techniques: Comparative genomic hybridization (CGH) is another new molecular cytogenetic technique that has recently been developed for detecting chromosomal imbalances in tumor genomes ***(38)***. CGH is based on two-color FISH. Equal amounts of differentially labeled tumor DNA and normal DNA are mixed together and hybridized, under conditions of Cot-1 DNA suppression, to normal metaphase spreads. In a single experiment, CGH identifies DNA gains and losses and maps these variations to metaphase chromosomes. DNA extracted from either fresh or frozen tissues, cell lines, as well as from formalin-fixed, paraffin-embedded samples is suitable for CGH ***(39)***. CGH becomes particularly advantageous when structural analysis of chromosomal changes in cancers are severely limited by their banding quality. Of note is that CGH is an effective screening method for describing and establishing a phenotype/genotype correlation in solid tumor progression. Several examples of chromosomal aberrations that define specific stages in tumor progression have already been established in brain, colon, prostate, cervix, and breast carcinogenesis ***(38)***.

   Cancer cytogenetics is often hampered by low mitotic indices, poor quality metaphase spreads, and the presence of complex marker chromosomes. A newly developed technique—multicolor spectral karyotyping (SKY)—may have the ability to overcome these obstacles (*see* **Fig. 4**). This technique combines Fourier spectroscopy, charge-coupled device (CCD) imaging, and optical microscopy to measure chromosome-specific spectra after FISH with differentially labeled painting probes ***(40,41)***. This technique was reported to be in excellent agreement with results from previously performed FISH experiments and banding analysis. Currently, work is underway to generate a multicolor banding pattern (bar code) of the human chromosome complement by using chromosome arm- and band-specific painting probes in order to identify intrachromosomal anoma-

Fig. 4. A SKY picture showing the marker chromosome (indicated by arrow) to be of chromosome 18 in origin. This marker could not be identified with certainty by G-banding.

lies. Therefore, it appears that SKY may be a very promising approach to the rapid and automatic karyotyping of neoplastic cells.

Chromosomal microdissection to obtain DNA and subsequent PCR generation of FISH probes is another powerful analytical tool. Microdissected chromosomal DNA can generate whole chromosome paint probes and band- or region-specific probes. This technique is particularly useful in identifying the

origin of chromosomes or chromosomal regions that cannot be conclusively identified by cytogenetics. The approach, combining PCR to produce probes from microdissected chromosomal DNA and subsequent FISH analysis, has been defined as micro-FISH ***(42,43)***.

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3

Comparative Genomic Hybridization for the Analysis of Leukemias and Lymphomas

Michael Baudis and Martin Bentz

1. Introduction

Cytogenetic methods have become increasingly important tools for both research in hematological malignancies and for the diagnostic workup of leukemias and lymphomas. The knowledge about specific chromosomal aberrations has been an essential prerequisite for the identification of pathogenetically relevant genes. Important examples are molecular genetic analyses of the breakpoint regions in chromosomal translocations, which resulted in the detection of protooncogenes such as *ABL* in chronic myeloid leukemia (CML) and acute lymphoblastic leukemia (ALL) [t(9,22)(q34;q11)], or *MYC* in Burkitt's lymphoma [t(8;14)(q24;q32); for a review *see* **refs. *1*** and ***2***].

A number of specific chromosomal aberrations are highly characteristic for certain subtypes of leukemia and lymphoma. The presence of a t(9;22)(q34;q11) or a BCR-ABL fusion, its molecular counterpart, is found in almost all cases of CML and serves as a diagnostic criterion to distinguish CML from other myeloproliferative disorders ***(3)***. Another example is the t(11;14)(q13;q32) characteristic of mantle cell lymphoma. The recognition of this chromosomal translocation was an important step for the general acceptance of mantle cell lymphoma as a distinct lymphoma entity ***(4)***. The close association between genetic aberrations and pathomorphological/clinical parameters has been further underscored by the inclusion of cytogenetic data in the most recent proposal for the classification of lymphoid neoplasms ***(5)***. Whereas to the present day most published studies have focused on the prevalence of specific aberrations in different tumor types, analyses in larger numbers of cases will determine whether or not these aberrations have prognostic value regarding treatment outcome or survival ***(5a)***. Indeed, specific chromo-

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somal aberrations have also been demonstrated to be of prognostic importance, especially in acute leukemias ***(6,7)***. Currently, several clinical trials are underway that use cytogenetic data to stratify patients into different risk groups ***(8,9)***. With further advances in cytogenetic and molecular cytogenetic methods, such correlations between genetic aberrations and the clinical course may become evident for other hematological malignancies, such as chronic lymphocytic leukemia ***(10)*** and non-Hodgkin's lymphoma ***(11,12)***.

Probably the single most important methodological development for tumor cytogenetics has been the introduction of fluorescence *in situ* hybridization, using genomic DNA probes (fluorescence *in situ* hybridization, FISH; *see* **refs. *13–15***). For this technique, nucleic acid probes for the detection of specific DNA sequences can be hybridized to both metaphase spreads and interphase nuclei of tumor samples, thereby creating the possibility of "interphase cytogenetics" ***(16)***: the number and/or distribution of the fluorescent signals provides information about the presence of numerical or structural genetic aberrations (for review *see* **ref. *17***).

Whereas FISH studies have greatly contributed to the understanding of tumor biology and the clinical relevance of specific chromosome aberrations in a variety of tumors ***(18,19)***, this approach has at least two major limitations. Because the target regions for the specific nucleic acid probes are small (approximately 30 kbp–1 Mbp), a preknowledge about possibly relevant chromosomal subregions or affected genes is required. Furthermore, in every experiment only a limited number of chromosomal regions can be investigated, resulting in the analysis of only a small portion of the entire tumor genome. In contrast, the more recently introduced approach of comparative genomic hybrididization (CGH; *see* **ref. *20***) allows a comprehensive analysis of chromosomal gains and losses without any preknowledge of affected chromosome regions.

The principle of CGH is outlined in **Fig. 1**. For this technique, basic principles of FISH are combined with the application of quantitative digital image

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Fig. 1. (*opposite page*) Schematic illustration of comparative genomic hybridization (CGH). **(1)** Genomic DNA derived from tumor tissue and from normal tissue (control-DNA) is labeled in a nick translation reaction using biotinylated or digoxigenin-conjugated nucleotides, respectively. **(2)** Tumor and control DNAs are hybridized simultaneously to denatured *normal* human chromosomes. **(3)** In the detection reaction, the modified nucleotides bound to the chromosomes are detected using appropriately conjugated fluorochromes, e.g., avidin-FITC and anti-digoxigenin-conjugated rhodamine, respectively. Note that the relative concentration of tumor and control DNA in the hybridization solution determines the resulting fluorescence pattern. **(4)** Depending on the relative sequence copy number of the test and control DNA, the differences of the fluorescence intensities can be visualized. Chromosome regions

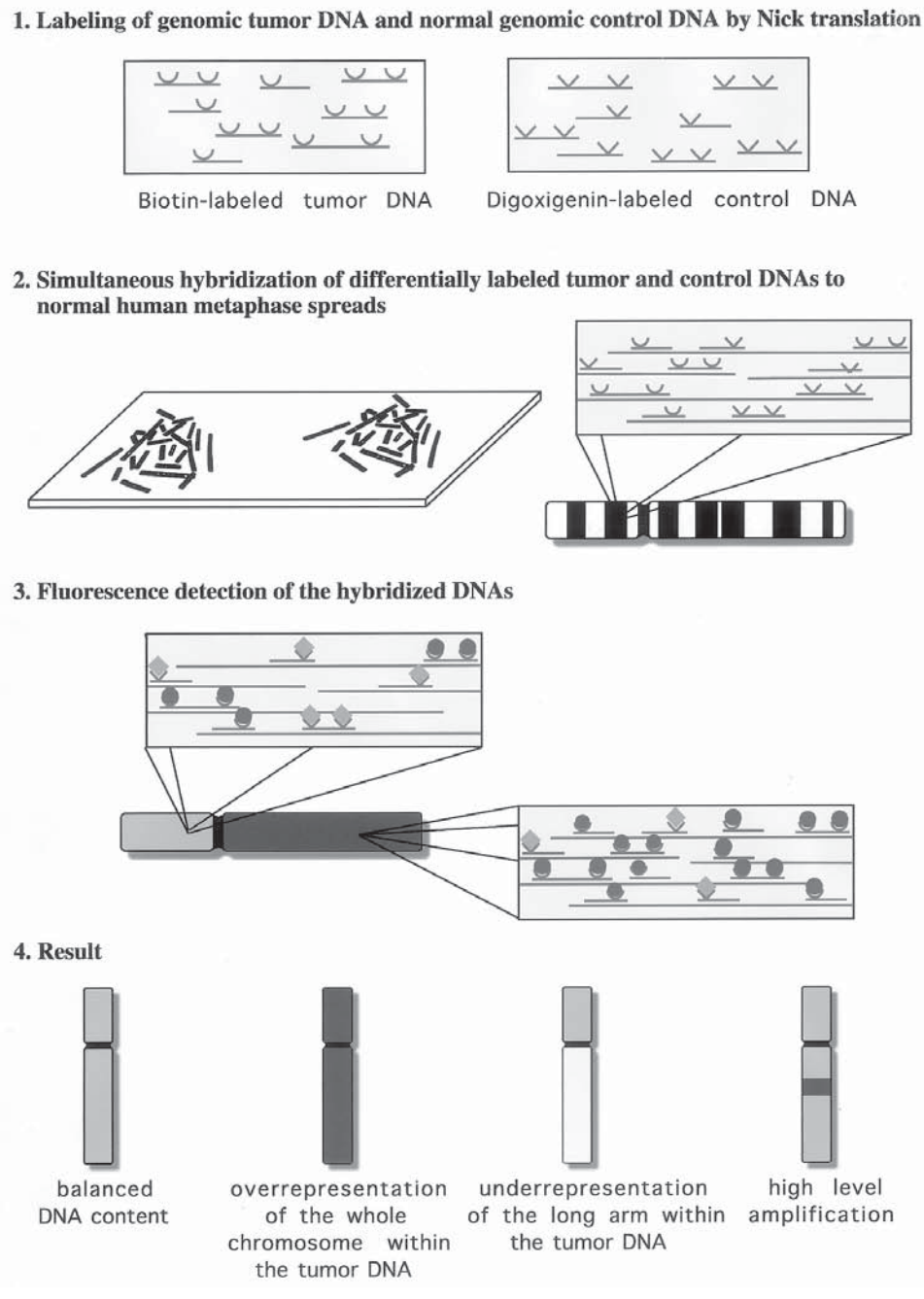

Fig. 1. (*continued*) with a balanced genomic content appear yellowish, whereas fluorescence color in regions overrepresented in the tumor genome is more green, and the fluorescence color in regions underrepresented in the tumor genome is more red. High-level DNA amplifications are visible as distinct, bandlike green hybridization signals.

analysis ***(20,21)***. In contrast to the classical FISH method, for which a well-defined DNA probe is hybridized to tumor cells with unknown genomic content, a reverse approach is used for CGH: whole genomic DNA with unknown genomic content derived from tumor tissue is hybridized to well-defined (i.e., normal) human metaphase spreads ("reverse painting"). This results in a more or less homogeneous staining of the normal metaphase chromosomes. If, however, certain chromosome regions are over- or underrepresented within the tumor DNA, this will result in a more (overrepresentation) or less (underrepresentation) intense staining of the respective regions on the normal chromosomes. The simultaneous hybridization of differentially labeled normal genomic DNA in the same reaction (derived from healthy human tissue, such as, e.g., a placenta or blood from a healthy donor; "control DNA") serves as internal control. Such an internal control is required to compensate for signal inhomogeneities caused by variabilities in the experimental conditions. Comparing the signal intensities of tumor and control DNAs allows the detection of chromosomal imbalances. For a reliable and reproducible quantitative analysis of fluorescence intensities, dedicated image analysis software applications are used.

Future progress in this field can be expected from the introduction of microarray technologies ("DNA chips"; for a review *see* **ref. *22***). For these applications, the matrix for the hybidization consists of an ordered set of DNA probes immobilized on a glass slide. RNA-based screening of gene expression by hybridization to immobilized cDNA probes has been demonstrated. However, hybridization of whole genomic human DNA to immobilized DNA probes has been hampered by the low probability of specific hybridization due to the complexity of the probe. Only very recently, a protocol has been published, that in principle allows the application of CGH to "biochips" consisting of glass slides with immobilized target DNAs ("matrix-CGH"; ***23***). Such target DNAs may consist of DNA of high complexity, e.g., like material from a whole chromosomal arm or single chromosomal bands or may even consist of short sequences specific for a gene of interest. For cytogenetic diagnostics in tumor samples, arrays containing samples of targets with known involvement in the given malignancy could be used to probe the tumor DNA of a patient in a single reaction for the existence of these aberrations. In this way, a disease-specific probe set could be created. In contrast to CGH to chromosomal targets, it can be envisioned that analysis of such "matrix CGH" experiments can be performed in an automated fashion. This would open completely new perspectives for cytogenetic diagnostics in clinical hematology and oncology. However, in the meantime, current CGH analyses require the following experimental steps:

1. Preparation of slides containing high-quality metaphase preparations of normal human chromosomes, which serve as a hybridization matrix.
2. Isolation of genomic DNA from tumor tissue.
3. Differential labelling of tumor and control DNA by incorporation of chemically modified nucleotides.
4. Simultaneous *in situ* suppression hybridization of tumor and control DNA.
5. Fluorescence detection of the hybridization reaction.
6. Image acquisition and digital image analysis.

Protocols for each of these steps are described in **Subheading 3.** Apart from digital image analysis, all these procedures are also used for other molecular genetic and/or cytogenetic applications. For CGH, however, special attention is required at several points within these protocols.

2. Materials

2.1 Preparation of Metaphase Spreads from Blood Lymphocytes

1. Heparinized blood from a healthy donor.
2. RPMI 1640 culture medium containing 20% fetal calf serum (FCS), 1% L-glutamine 200 m*M*), 1% penicillin (5000 IU/mL), 1% streptomycin (5000 µg/mL) and 1.5% PHA
3. Precleaned microscope slides.
4. Incubator with CO_2-enriched atmosphere.
5. 1% Colcemid solution.
6. Hypotonic solution: 40 m*M* KCl, 20 m*M* HEPES, 0.5 m*M* ethyleneglycol-tetraacetic acid (EGTA); adjusted to pH 7.0 using 1*M* KOH.
7. Fixative (three parts methanol, one part acetic acid).
8. Cooled centrifuge.
9. Moist chamber.
10. Coplin jars containing ethanol in rising concentrations (70, 90, and 100%).

2.2. Isolation of Genomic DNA from Paraffin-Embedded Tissue Samples

1. Microtome (when processing paraffin-embedded tissue).
2. Xylol (100%).
3. Methanol (100%).
4. Sodiumisothiocyanate (NaSCN, 1 *M*).
5. DNA isolation buffer: 75 m*M* NaCl, 25 m*M* ethylenediaminetetraacetic acid (EDTA), 0.5% Tween-20, pH 7.5.
6. Proteinase K stock solution (20 mg/mL).
7. Phenol (buffered, pH 7.5).
8. CIA (24 parts chloroform : 1 part isoamylalcohol).
9. Chloroform (100%).

10. Isopropanol (100%).
11. 3 *M* Sodium acetate, pH 5.2.
12. Ethanol (biograde, 70%).
13. TE buffer: 10 m*M* Tris-HCl, 1 m*M* Na_2 EDTA, pH 8.0.
14. Sephadex G50 columns.
15. Photometer for measurement of DNA concentration.

2.3. Nick Translation

1. 10X Reaction buffer: 0.5 m*M* Tris-HCl, pH 8.0, 50 m*M* $MgCl_2$, 0.5 mg/mL bovine serum albumin (BSA).
2. 0.1 *M* β-Mercaptoethanol.
3. 10X Nucleotide solution containing 0.5 m*M* dATP, 0.5 m*M* dCTP, 0.5 m*M* dGTP, 0.5 m*M* biotin 16 dUTP, and 0.12 m*M* dTTP for biotinylation of the probe DNA; for digoxigenin labeling, digoxigenin-11-dUTP is included in the stock solution (0.5 m*M* dATP, 0.5 m*M* dCTP, 0.5 m*M* dGTP, 0.125 m*M* digoxigenin-11-dUTP and 0.375 m*M* dTTP). For direct labeling, similar nucleotide solutions containing nucleotides directly coupled to a fluorescent dye can be used.
4. Polymerase I (*Escherichia coli*).
5. DNAse I solution: 3 mg in 1 mL 0.15 *M* NaCl and 50% glycerol.
6. Agarose mini gel (1–2%) and electrophoresis equipment.
7. Gel-loading buffer; size marker.
8. Ethidiumbromide solution (0.5 µg/mL).
9. EDTA (0.5 *M*).
10. Sodium dodecyl sulfate (SDS) (10%).
11. Sephadex G50 spin columns.

2.4. Hybridization (*see* Note 1)

1. Labeled test and control DNAs.
2. Human C_0t_1 DNA.
3. Ethanol (biograde, 70 and 100%, ice cold).
4. Deionized formamide (conductivity < 100 µS).
5. Hybridization buffer (4X standard saline citrate (SSC), 20% filtered dextran sulfate).
6. Slides containing metaphase preparations.
7. Denaturation solution (70% deionized [<100 µS] formamide, 2X SSC, 50 m*M* sodium phosphate, pH = 7.0).
8. Coplin jars containing ice-cold ethanol in rising concentrations (70, 90, and 100%).
9. Coverslips (18 ×18 mm); rubber cement.
10. Water bath (70°C), oven (60°C), moist chamber, incubator (37°C).

2.5. Hybridization Detection

1. Washing solutions:
   a. 50% formamide, 2X SSC, pH 7.2
   b. 0.1X SSC, pH 7.2

c. 4X SSC, 0.1% Tween-20, pH 7.2
d. 2X SSC, 0.05% Tween-20
2. Blocking solution: 4X SSC, 3% BSA
3. Detection solution: 4X SSC, 1% BSA, 0.1% Tween-20
4. Avidin-fluorescein isothiocyanate (FITC).
5. Anti-digoxigenin-rhodamine.
6. Counterstaining solution: 2X SSC, 200 ng/mL 4′-6-diamidino-2-phenylindole-2 HCl (DAPI)
7. Antifade solution: 1,4-diazobizyclo [*see* **Subheading 2.2., step 2**) octane (DAPCO); or other commercially available antifade solutions
8. Coplin jars, normal and with light protection.
9. Heatable shaking water bath.
10. Cover slips, moist chamber, incubator (37°C).

3. Methods

3.1. Metaphase Preparation for Comparative Genomic Hybridization (CGH) Experiments

The generation of slides containing metaphase spreads of constant high quality is one of the most important prerequisites for CGH experiments (*see* **Note 2**). This is a critical step, and even in experienced hands, batches of slides may be produced, that are of insufficient quality. Most importantly, metaphase spreads should allow an optimum hybridization reaction resulting in a homogeneous fluorescence painting of all chromosomes. This can only be achieved on slides without any residual cytoplasm and without notable background signals. Ideally, the chromosomes should be at least of intermediate size and there should be little overlap of chromosomes. Using the following protocol, about 100–200 slides containing metaphase spreads of high quality can be prepared (*see* **Note 3**).

1. Five milliliters of heparinized blood from a healthy donor are added to 45 mL of RPMI 1640 culture medium.
2. One day before harvesting the cells, precleaned microscope slides are transferred in 100% ethanol and washed overnight. Immediately before the cells are dropped, they are dipped several times into water and dried.
3. The cell suspension is incubated for exactly 72 h at 37°C in an atmosphere of 5% O_2 (*see* **Note 3**).
4. After this period, 500 µL of an 1% colcemid solution is added (final concentration: 0.1 µg/mL) and the cells are incubated for another 20 min.
5. The solution is transferred into a 50-mL polystyrol tube and the tube is spun at 200*g* for 5–10 min.
6. The supernatant is removed, the pellet is resuspended by gently shaking in the remaining medium, and 2 mL of hypotonic solution are slowly added.

7. The tube is filled with hypotonic solution and incubated for 15–20 min at 37°C. Afterward, it is spun at 200*g* for 10 min.
8 The supernatant is removed, the pellet is again resuspended in the remaining solution, and some drops of ice-cold fixative (kept on ice) are slowly added; then slowly, drop by drop, more ice-cold fixative is added up to a total of 8–10 mL. During this whole step, the tube should be shaken gently. It is essential to take sufficient time for this important step, which may be crucial for the success of the preparation!
9. The tube is spun for 10 min at 200*g* in a cooled centrifuge (4°C). The supernatant is then removed and again ice-cold fixative is added equally slowly as in **step 8**. This step is repeated at least once.
10. The tube is incubated on ice for 30–60 min. Afterward, it is spun again in the cooled centrifuge and the pellet is resuspended. The cycle of very slowly adding fixative-spinning-resuspending is repeated at least five times.
11. After the last cycle, the pellet is resuspended in about 1–2 mL fixative solution.
12. Single drops of the suspension are dropped from a distance of about 50 cm on the prewashed slides, which are kept in a moist environment, e.g., above a heated water bath (95–100°C). One drop will provide a sufficient number of metaphase spreads for a CGH experiment. However, the quality of the metaphase spreads and cell concentration have to be checked before continuing the procedure. If the cell concentration is too high, more fixative is added. If it is too low, the suspension is spun once more and the pellet is resuspended in a smaller volume of fixative. If considerable amounts of residual cytoplasm are present, additional rounds of washing in fixative may be helpful.
13. The slides are transferred into a moist chamber for about 5 min; thereafter, they are air dried and kept at room temperature for at least 1 d.
14. Dehydration of the slides is achieved in a series of ethanol (70, 90, and 100% for 5 min each).
15. Afterward, the slides may be used directly for CGH experiments within a few days. Alternatively, slides can be be sealed in plastic bags containing a drierite to prevent water condensation and stored at –80°C for many months.

After a new batch of slides is prepared, checking the quality for CGH is mandatory. This can be done by a test hybridization using DNA of known superior quality. If no satisfactory hybridization can be achieved, the whole batch of slides should be discarded. In our experience, it is more convenient to prepare a new batch of slides than trying to cope with slides of inadequate quality. Due to variations of slide quality within a batch, it is also necessary to check all slides by phase contrast microscopy prior to using them for hybridization.

3.2. Isolating Genomic DNA from Paraffin-Embedded Tissue Samples

Genomic DNA can be extracted from a variety of sources. High-quality DNA is easily obtained from fresh or frozen tissue samples. For such DNA

sources, standard proteinase K digestion and subsequent phenol extraction protocols may be applied ***(24)***, but it is also convenient to use one of the commercially available kits (addressed in other chapters of this volume). However, in many cases, fresh or frozen tissue material is not available. Especially for the analysis of lymphomas (and solid tumors), very often paraffin-embedded, formalin-fixed tissue samples are the only possible sources of DNA. Due to the fixation with formaline, DNA quality in these samples has been impaired. This fixation process results in the degradation of the DNA and in a crosslinking between DNA and proteins ***(25)***. Because cytogenetic analysis of paraffin-embedded samples is one of the major advantages of CGH in comparison to other cytogenetic methods, we describe our currently used protocol for the isolation of DNA from paraffin-embedded tissue blocks as follows.

1. Remove the paraffin surrounding the tissue. Thereafter, thirty to sixty 6-µm-thick tissue sections are cut using a microtome and placed in an Eppendorf tube. The exact number of slices depends on the surface area of the used material. With this protocol, 15–30 mg of tissue will result in 50–100 µg of DNA.
2. For dewaxing, fill the Eppendorf tube with xylol. After the xylol is shaken for 10 min, it is removed. This step is repeated once and followed by washing with methanol.
3. The slices are lyophilized in a vacuum centrifuge and thereafter incubated overnight in 1 *M* NaSCN. This may minimize crosslinking of the DNA with denatured proteins ***(26)***.
4. After removing the NaSCN, which is facilitated by centrifugation of the tubes to precipitate the material, briefly wash the material twice at room temperature using an isolation buffer.
5. Remove the buffer and add 1.5 mL of fresh isolation buffer containing 0.5 µg/mL proteinase K. Incubate the solution for about 72 h at 55°C in a shaking incubator or a water bath. After adequate digestion, no solid particles should be visible. If this is not achieved, more proteinase K may be added.
6. The solution is taken out of the incubator, an equal amount of buffered (pH = 7.5) phenol is added and gently mixed for about 20–30 min in a shaker at room temperature. Afterward, the tube is centrifuged at high speed for 10min (e.g., at 10,000*g* when using Eppendorf tubes, room temperature) and the watery phase is collected using a small-bore pipet. This procedure is repeated once.
7. The same procedure is applied once using a mixture of 50% phenol/50% CIA. This is followed by one round of washing in 100% chloroform.
8. After the last washing step, the watery phase is put in a fresh tube and precipitated by adding 2 vol isopropanol and 1/20 vol 3 *M* sodium acetate (pH = 5.2). Due to the degradation of the DNA derived from paraffin-embedded tissue, no visible DNA thread is expected. Spin the tube at high velocity (e.g. 10–15,000*g* for 10 min when using Eppendorf tubes) to allow sedimentation of the DNA.
9. The liquid is carefully removed. Ice-cold ethanol (70%) is added and the precipitate is washed by vortexing before spinning the tubes again.

10. Again, the supernatant is discarded. The pellet is lyophilized using a vacuum centrifuge.
11. An appropriate volume (50–200 µL) of TE buffer is added. The pellet is dissolved overnight at 4°C.
12. For optimum results, the solution should be cleaned from salts and other impurities by elution through Sephadex G50 columns. The concentration of the DNA in the solution is measured using a photometer and the purity is estimated by calculating the ratio of the absorption at 260 and 280 nm, respectively (*see* **Note 4**). If the ratio is below 1.8, this may indicate a high degree of contamination by residual proteins. In this case, an additional phenol extraction may be required. The length of the DNA fragments, which is an indicator of the degree of degradation, can be estimated by running an agarose minigel.

The quality of DNA is directly correlated to fixation times and the fixatives used. Unfortunately, in most cases, no information regarding the exact fixation conditions are available. However, in our experience, samples that were exposed to formalin for as long as 48 h can be used for CGH if a buffered formalin solution was used.

3.3. Probe Labeling

The labeling of DNA for the use in fluorescence *in situ* hybridization (FISH) experiments can be performed in several ways. The commonly applied methods use chemically modified nucleotides conjugated to reporter molecules or fluorescent dyes. These modified nucleotides are incorporated into the probe DNA. With both nick translation and primer extension ***(27)***, similar labeling efficiencies can be achieved. However, we prefer the use of nick translation, because this technique allows the convenient adjustment of the probe size ***(28)***, which is critical for a successful CGH experiment. We use an indirect labeling approach: the tumor DNA is labeled with biotin-conjugated nucleotides, whereas for the control DNA digoxigenin-labeled nucleotides (nt) are used. Direct labeling with fluorescent dyes is preferred by some groups ***(29)***. In our hands, such directly labeled DNA probes result in a homogeneous painting of all chromosomes; however, the signal intensity is reduced when compared to indirectly labeled probes.

The protocol for a 100-µL reaction volume sufficient for labeling 2 µg of DNA is described in the following list. Because of the limited solubility of DNA in aequous solutions, the DNA concentration of the labeling solution must not exceed 20 ng/µL.

1. Ten microliters of reaction buffer, 10 µL nucleotide solution, 10 µL β-mercaptoethanol, the appropriate volume of the probe solution containing 2 µg of DNA and dH_2O for a total volume of 94 µL are transferred into an Eppendorf

tube. Thereafter, 3 µL polymerase I and 3 µL of a dilution of DNAse I (1 : 1000 in ice-cold water) is added.
2. The reaction tube is incubated for 90 min in a water bath at 15°C. The appropriate time may vary depending on the quality of the DNA and the activity of the enzyme batches used. After incubation, the tubes are put on ice again to interrupt the reaction.
3. The length of the fragments is checked by gel electrophoresis. Five to 10 µL of the solution are combined with 4 µL of gel-loading buffer and boiled in a water bath for 2–3 min. The gel is loaded onto an agarose minigel, adding a suitable size marker in an extra lane. A quick run is performed applying about 15 V/cm for about 30 min. The DNA is visualized by staining in the gel with ethidium bromide, and a photograph under ultraviolet illumination is taken.
4. The probe fragments visible as a smear should be in the range of 500–1000 nt (*see* **Note 5**). If the probe size is not within this range, proceed as follows:
   a. Probe smear is smaller than 500 bp and part of the probe is smaller than 100 bp: discard the reaction solution and start the labeling reaction again.
   b. Probe size is larger than 1000 bp: more DNAse I is added and digestion is allowed for another 15–30 min. The fragment length is checked again. Digestion times and enzyme concentrations have to be individualized for each single probe and each batch of enzymes.
   c. Probe is not or almost not digested: check quality of the enzymes used; if enzymes work well with other probes, most likely the DNA probe is of low purity. It should be purified and the nick translation should be started again.
5. If the probe size is within the desired range, 2 µL of EDTA (0.5 *M*) and 1 µL of SDS (10%) are added and the solution is incubated at 68°C for 10 min to inactivate the enzymes.
6. In a final step, the probes are cleared of unincorporated nucleotides by gel filtration using Sephadex G 50 spin columns. The final concentration of the labeled DNA is approximately 20 ng/µL. The labeled probe can be stored at –20°C.

3.4. Hybridization of the Probe to Slides Containing Metaphase Chromosomes

CGH as well as other FISH techniques using probes of high complexity has been made possible by the development of chromosomal *in situ* suppression (CISS) protocols ***(13–15)***. Without such a suppression, hybridization would not result in a specific fluorescence pattern because of the high content of interspersed repetitive sequences (IRS) in the genome ***(30)***. Binding of labeled probe DNA to these IRS is minimized by combination of the DNA probe with excess amounts of unlabeled human C_0t_1 DNA and inclusion of a preannealing step.

1. One microgram each of labeled test and control DNAs as well as 70 µg of human C_0t_1 DNA are combined in an Eppendorf tube. The DNA is precipitated by add-

ing 2 vol of ice-cold ethanol and 1/20 vol of 3 *M* sodium acetate (pH = 5.2) and by subsequent incubation for 30 min at –70°C .

2. The tubes are spun at 10–15,000*g* and 4°C for 10min. The supernatant is discarded and the pellet is washed briefly with 70% ethanol before spinning again. After the supernatant is removed, the pellet is lyophilized using a SpeedVac.
3. The pellet is resuspended in 6 µL deionized formamide by gently vortexing for 30 min. Thereafter, 6 µL hybridization buffer is added and the solution is agitated for another 30 min.
4. The DNA is denatured for 5 min at 75°C. Subsequently, the tubes should be cooled for some minutes on ice, before preannealing of the probes is performed for about 20 min at 37°C. At this point, the tube can be kept on ice.
5. While performing the steps described above, prepare the slides containing the metaphase spreads in parallel. When using frozen slides, it is important to thaw them slowly, thereby avoiding water condensation. Slides stored at –80°C are first transferred to –20°C for at least 30 min. Then they are kept for at least another 30 min at +4°C, before unpacking them and keeping them at room temperature. Usually about twice the number of slides as needed for the CGH experiments are thawed to allow for a selection.
6. The slides are checked with a phase contrast microscope and the best slides are selected based on low visible background, size, and spreading of the metaphase chromosomes (*see* **Subheading 3.1.**). The area with the highest density of metaphase spreads is marked on the backside of the slide using a diamond pen.
7. Prior to denaturation, the slides are heated for 15–60 min in an oven at 60°C to prevent dropping of the temperature of the denaturation solution when adding the slides.
8. A Coplin jar is filled with denaturation solution and heated in a water bath (70°C). Slide denaturation is another critical step. Therefore, the temperature of the denaturing solution has to be exactly at 70°C.
9. The slides (not more than three at a time) are transferred into the denaturation solution. The denaturation should be performed for exactly 2 min (*see* **Note 6**). During this time, the temparature of the solution is monitored and kept between 69.5 and 70.5°C. Longer denaturation times or higher temperatures may impair the integrity of the chromosomes, whereas shorter denaturation times or lower temperatures will decrease the hybridization efficiency.
10. After 2 min, the slides are transferred immediately to Coplin jars containing ice-cold ethanol in rising concentrations (70, 90, and 100%) for 4 min each. After air-drying, the slides are ready for hybridization.
11. Transfer the dry slide to a heating block (42°C). The hybridization mixture is applied to the selected area, a small (18 × 18 mm) coverslip is put onto the droplet and the edges of the coverslip are sealed with rubber cement.
12. Afterward, the slides are incubated in a moist chamber at 37°C for about 2 d.

In contrast to the foregoing protocol, many groups use smaller amounts of tumor-, control-, and C_0t_1-DNAs ***(26,29)***. Although such an approach has obvious advantages (less tumor material and smaller amounts of the costly

C_0t_1-DNA are required for a hybridization), disadvantages include prolonged hybridization times (3–5 d), and less homogeneous hybridization patterns.

3.5. Detection Procedure

It is essential that the slides never get completely dry during the whole detection procedure. Therefore, care should be taken that exchanging of solutions is performed rapidly.

1. The slides are taken out of the chamber and the rubber cement is carefully removed. Slides are then transferred into a Coplin jar containing washing solution A, which is kept at 42°C in a shaking water bath. After 10 min, the solution is removed and again, the jar is filled with washing solution A. At this step, the coverslips should slide off. The slides are washed three more times in solution A (42°C, for 5 min each).
2. Thereafter, slides are washed three times for 5 min each in washing solution B (60°C).
3. After flow-off of the solution, 200 µL of blocking solution are applied onto the slides and covered with large (24 × 60 mm) coverslips. The slides are incubated for at least 30 min in a moist chamber at 37°C.
4. In the meantime, the detection solution containing the fluorochromes is prepared. For each slide 200 µL of detection solution is used, and 1 µL of avidin-FITC (final concentration: 5 µg/mL) as well as 3 µL of anti-digoxigenin-rhodamine (final concentration: 6 µg/mL) are added.
5. After the coverslips are removed, 200 µL of detection mixture are applied to each slide. Starting with this step, light exposure of the slides should be minimized. After application of the coverslips, the slides are transferred immediately into a darkened moist chamber, where they are incubated for 30 min at 37°C.
6. After the coverslips are removed, the slides are transferred to a darkened Coplin jar containing washing solution C and washed three times for 5 min each using a shaking water bath (temperature: 42°C).
7. The slides are transferred into a darkened Coplin jar containing counterstaining solution. They are incubated for 20 min at room temperature while the solution is shaken gently.
8. The slides are washed for 2 min in solution D. Each slide is taken out of the jar, the liquid on the slides is drained and 15–20 µL of an antifade solution is applied to the hybridization area to minimize the bleaching of the fluorescence signals; then a medium sized coverslip (24 × 50 mm) is applied. The slides can be stored in suitable boxes at 4°C for many months.

3.6. Image Acquisition and Analysis

3.6.1. Requirements and Equipment

For image aquisition, an epifluorescence microscope of research standard is needed. Because signal intensity and homogeneity of the illumination can be

critical for CGH evaluation, we recommend the use of a carefully adjusted 100 W mercury lamp as the light source. Even more importantly, appropriate narrow bandpass filters matching the spectra of the used fluorochromes have to be selected. In order to minimize the pixel shift of the different images, an automatic filter wheel or slider containing single band-pass excitation filters with a stationary triple band-pass emission filter may be advantageous. If filter blocks requiring the changing of both excitation and emission filters are used, correction of the shift prior to quantitative image analysis—either manually or by an algorithm implemented in the analysis software—is mandatory.

For the establishement of the CGH method, cooled charge-coupled device (CCD) cameras, which allow aquisition of microscopic images with superior sensitivity, had been used. These cameras still show an advantage concerning the visual appearance of the recorded images; however, video cameras, which are far less expensive, have shown to be absolutely appropriate for CGH. As only signal intensities have to be measured, black-and-white cameras are recomended for this purpose (*see* also **ref. *31***).

Recently, systems combining the essential parts of hard- and software have become commercially available by several suppliers, most of which allow image acquisition and quantiative image analysis at high quality.

3.6.2. Principles of Quantitative Image Analysis

Although gross chromosomal aberrations can be easily detected by visual inspection of the metaphase spreads, CGH strongly depends on software supported quantitative image analysis. Most of the steps required for the quantitative analysis of CGH images is performed by currently available software without any user interaction. However, for the correct interpretation of CGH results, some knowledge about the principles of image analysis is required.

Briefly, the following steps have to be performed (*see* also **refs. *30–34***):

1. Chromosome segmentation: areas of the images that represent chromosomes are defined.
2. Background subtraction: the presumed value of the background fluorescence is subtracted from the whole images, resulting in a close approximation of the specific intensities of the hybridization signals on the chromosomes.
3. Definition of the chromosomal axis.
4. Measuring the fluorescence intensity values of control and testing the DNA for each pixel inside the chromosome mask. For calculating the fluorescence ratio profiles (*see* **Step 6**), the value for each pixel on the chromosomal axis is defined by the median values of all pixels perpendicular to the respective pixel on the chromosomal axis. This value is normalized, usually under the assumption that the most frequently occuring ratio value within an experiment (“modal value”) equals a ratio value of 1.0.

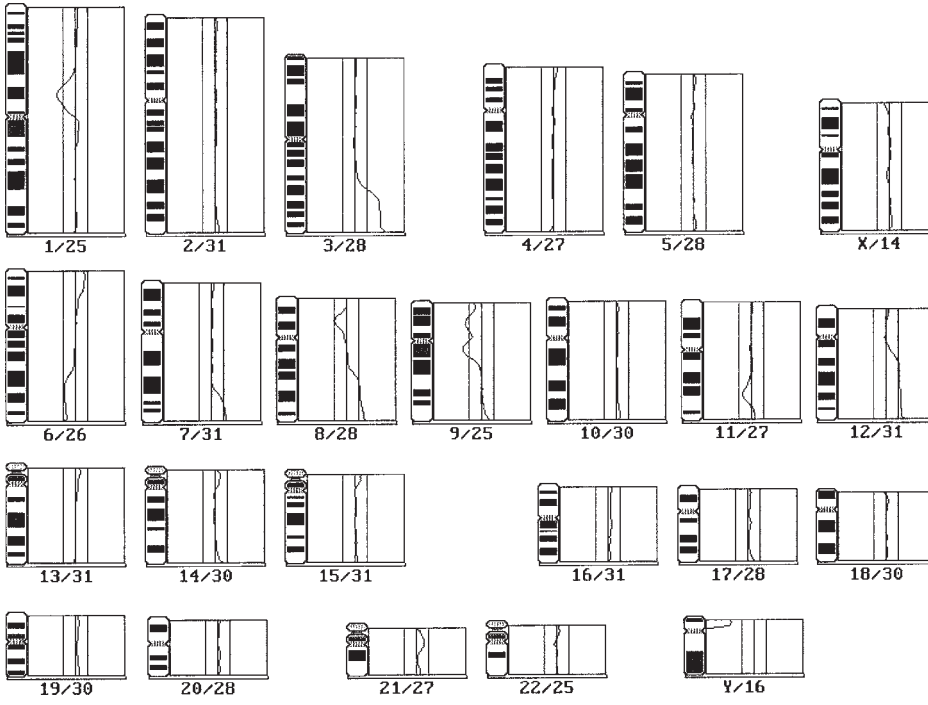

Fig. 2. Average ratio profile of the case shown in **Fig. 2**. The ratios of FITC to rhodamine fluorescence are plotted along the chromosomes. The central line indicates a ratio value of 1.0, whereas the other lines are used to assess genomic copy number differences. The lines to the right indicate a ratio value of 1.25, and the lines on the left indicate a value of 0.75, which are used as thresholds for the diagnosis of an overrepresentation and an underrepresentation, respectively.

5. Chromosome identification: in an interactive step, chromosomes have to be identified by the user with an option in some of the systems to presort the chromosomes. Overlapping chromosomes as well as artefacts have to be excluded in this step.
6. Calculation of the average ratio profiles: For each pixel value on the axis of each chromosome, the mean value of all chromosomes of a specific type (usually 15–25) is calculated. These mean values are plotted along chromosome ideograms as so-called "average ratio profiles" (*see* **Fig. 2**).

Based on these average ratio profiles, the CGH result is determined. Threshold values are defined for gains and losses of chromosomal regions. To the present day, fixed threshold values have been used by most investigators. Most frequently, a ratio value over 1.25 has been defined as indicative for an overrepresentation of genetic material, whereas values under 0.75 depicted losses of genetic material. These values have been extensively validated by

banding analysis (*see*, e.g., **refs. *35*** and ***36***) and interphase cytogenetics (*see* e.g., **refs. *37*** and ***38***). Remember that some chromosomal regions (telomeric portion of chromosome arm 1p, centromeric regions of chromosomes 1, 9, 16, and 19 as well as chromosome Y) are excluded from quantitative analysis. This is due to the extremely high content of repetitive sequences in these regions.

In order to increase the sensitivity of CGH, several groups have tried to use statistical threshold values (***32*** and ***38***). For such an approach, the calculation of the probability of a ratio value to differ from the mean ratio rather than rigid threshold values is applied. Certainly, more data with such statistical thresholds are required to safely estimate the sensitivity and the specificity of these criteria.

4. Notes

Although CGH experiments consist of a large number of steps, there are a few points, which, in our experience, are particularly often responsible for an insufficient result. The importance of these steps has already been emphasized in **Subheading 3.** For the sake of clarity, they are listed again at this point:

1. Troubleshooting: After hybridization, bright fluorescence signals and a homogeneous staining pattern of all chromosomes should be visible. In experiments of adequate quality, gross aberrations, e.g., gains or losses of whole chromosomes, can be detected directly under the epifluorescence microscope without the use of any digitized image analysis device. For investigators less experienced in CGH, also chromosome X can serve as a control of hybridization quality, if control DNA derived from a male has been used: under these circumstances, the hybridization signal of chromosome X should be markedly weaker than the signals of the other chromosomes, which are present in two copies within the (normal) control DNA. If this is not the case, the quality of the experiment is insufficient. Other hallmarks of poor hybridization quality are high background signals, weak hybrization signals and an inhomogeneous, speckled hybridization pattern. If repeated CGH experiments are of inferior quality, a systematic mistake at one of the key steps has to be suspected. Such a mistake can be identified by the additional usage of reference material for each procedure. The efficiency of the nick translation can be checked by DNA of known good quality that is labeled in parallel. Also, a dot blot assay can be performed, which allows the assessment of the amount of incorporated labeled nucleotides in the DNA. The success of the hybridization reaction can be validated by hybridizing an additional slide in parallel with labeled DNA, which has shown good results in previous experiments. Problems with the batch of fluorochromes may be present if only one color shows a weak hybridization signal. In cases with low signal intensities, in principle an additional signal amplification step may be considered ***(39)***. Although this leads to stronger hybridization signals, both background hybridization and signal granularitiy are increased. Therefore, signal amplification is not recommended. It is well to remember that aberrations reliably detected by

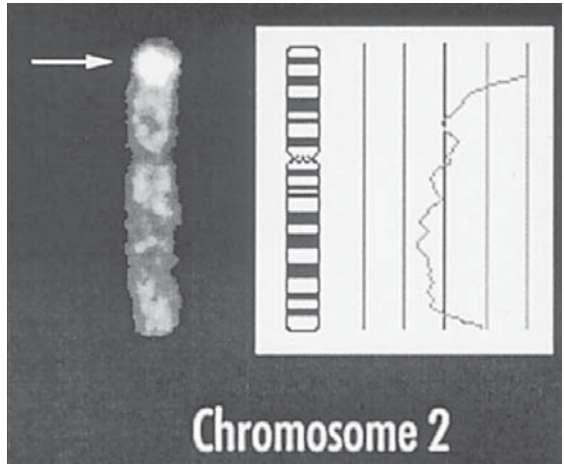

Fig. 3. High-level DNA amplification. Gray-level image of chromosome 2 after hybridization with DNA from a case with B-cell lymphoma. An intense, bandlike hybridization signal mapping to chromosomal bands 2p23–24 is visible (arrow). This is the chromosomal localization of the *N-MYC* protooncogene. In this case, a more than 100-fold amplification of this gene was demonstrated by Southern blot analysis ***(47)***.

CGH are those present in >50% of the cells in the analyzed sample. Therefore, it is mandatory that in cases with normal CGH karyotypes, the tumor cell content be carefully checked. In addition, aberrations must exceed a threshold size in order be detected. Whereas high level amplifications present in >20 copies can be detected down to a size of 100 kbp (***40***; *see* **Fig. 3**), reliable detection of a deletion is generally not feasible if the deleted segment is <10 Mbp ***(41)***.

2. Analysis of small tissue samples and microdissection: CGH strongly depends on a high tumor cell content within the analyzed sample. In certain hematological malignancies, e.g., multiple myeloma, hairy cell leukemia, primary extranodal lymphomas, or Hodgkin's disease, it may be difficult to obtain tissue samples in which the majority of cells are tumor cells. For such cases, the malignant cells have to be enriched prior to DNA isolation. One way to achieve such an enrichment is by fluorescence-activated cell sorting. Another way involves the microscopic dissection of frozen or paraffin-embedded tissue sections. This approach has been successfully applied to analyze primary large B-cell lymphomas of the gastrointestinal tract ***(32)***. In this lymphoma entity, tumor areas are often intermingled with normal gastric mucosa. In order to achieve a high tumor cell content, serial sections were cut from frozen tissue blocks. Every fifth section was stained to identify the area of highest tumor cell content. On the unstained slices in between, the localization of such tumor areas was reconstructed. These areas were dissected from the glass slides and the tissue pieces were collected in an Eppendorf tube. Afterward, genomic DNA was extracted and used for CGH

experiments. Microscopic dissection of tumor areas can even be used when the limited quantity of material precludes the isolation of sufficient DNA for CGH experiments. Under such circumstances, CGH can be combined with universal PCR protocols, which allow the representative amplification of minute amounts of DNA. Examples are the so-called degenerate oligonucleotide-primed PCR (DOP-PCR) ***(42)*** or sialic acid PCR (SIA-PCR) ***(43)*** protocols. Using these protocols, CGH can be performed with DNA obtained from only one tissue section (**refs. *44*,*45***). Currently experiments are underway, which aim to use single cells or collections of small numbers thereof to perform CGH experiments.

3. Preparation of high-quality metaphase spreads: It is important to incubate the cultures exactly for 72 h before adding the colcemide. Even more importantly, the fixation step needs to be done with utmost care. Time has to be taken to add the fixative very slowly.
4. High purity of the DNA: If the OD_{260}/OD_{280} ratio is below 1.8, an additional purification step is advisable.
5. Probe size after nick translation: It is essential that the size of the probe fragments in the range between 400–1000 bp. Larger probe sizes will result in large fluorescent signals outside the chromosome. Smaller probe fragments will lead to an unspecific binding of the labeled DNA.
6. Denaturation of the slides: It is essential that denaturation time is exactly 2 min and that the temperature of the denaturation solution is between 69.5°C and 70.5°C during the entire denaturation. Furthermore, the conductivity of the formamide used for the denaturation solution has to be below 100 µS.

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4

Spectral Karyotyping (SKY) of Hematologic Malignancies

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1. Introduction

1.1. Cytogenetic Analysis of Hematological Malignancies

The discovery of the Philadelphia chromosome in chronic myeloid leukemia (CML) by Novell and Hungerford in 1960 ***(1)***, the subsequent clarification of this chromosomal abnormality as a reciprocal translocation t(9;22)(q34;q11) by Rowley in 1973 ***(2)***, the identification of the genes involved at the translocation breakpoints ***(3,4)***, and ultimately the demonstration of the leukemogenic activity of the resulting fusion product ***(5)***, represent hallmarks for our understanding of malignant diseases as genetic disorders. The elucidation of the Philadelphia translocation emphasizes the importance of cytogenetic analysis of hematologic malignancies. Clarification of this chromosomal aberration as a reciprocal translocation became only possible after the development of cytogenetic banding techniques by Caspersson et al. in 1970 ***(6)***. Chromosome banding analysis revealed numerous nonrandom chromosomal aberrations, particularly balanced translocations in leukemias and lymphomas, e.g., the translocation t(8;21)(q22;q22) in acute myeloid leukemia (AML) first described by Rowley et al. in 1973 ***(7)***. These balanced translocations were shown to be of etiologic as well as diagnostic, prognostic, and therapeutic importance. They result in an altered gene function by two main mechanisms: (1) sequences of, in most instances, a transcription factor or receptor tyrosine kinase gene are fused to a normally unrelated gene, creating specific fusion proteins with oncogenic properties, and (2) protooncogenes are repositioned to

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the vicinity of promoter/enhancer elements of the immunoglobulin- or T-cell receptor genes, thereby initiating their activation ***(8)***.

Cloning genes located at the breakpoints and functional analyses contributed to our understanding of their role in malignant transformation, as well as to their involvement in the orchestration of normal hematopoiesis ***(8)***.

Despite the relevance of chromosomal banding techniques, the quality of chromosome preparations from malignant cells can impair the resolution of conventional cytogenetic analysis, hence the comprehensive description of complex karyotypes. The development of new molecular cytogenetic techniques like fluorescence *in situ* hybridization (FISH) has been extremely beneficial for the analysis of hematologic malignancies (*see* Chapter 2). For instance, the translocation t(12;21)(p13;q22) was first detected by chromosome painting ***(9)***. This translocation is difficult to discern by G-banding analysis because the telomeric segments of 12p and 21q are indistinguishable by their banding pattern. However, the t(12;21) is the most common genetic abnormality occurring in pediatric lymphoid leukemias of B-lineage (22%; **ref.** ***10***) and the presence of this translocation indicates a good prognosis independent of other features associated with a favorable course in acute lymphocytic leukemia (ALL) ***(8,10)***. The detection of chromosomal aberrations at diagnosis has become essential for risk-adapted therapy in childhood as well as adult acute leukemia (*see* review in **refs.** ***11*** and ***12***).

In lymphomas, a number of nonrandom chromosomal abnormalities is also associated with clinical, morphologic, and immunophenotypic features, but this correlation is better understood in acute leukemias than in any other hematological malignancy. For example, consistent genetic markers have not been identified for every currently recognized clinical entity of non-Hodgkin's lymphomas (NHL) ***(13)***.

Thus far, cytogenetic analysis has primarily relied on banding techniques. Important progress in cytogenetic analysis of hematological malignancies has been made following the introduction of FISH. But this technique is not suitable as an initial screening tool because not all chromosomes can be visualized in a single experiment. Visualization of all human chromosomes in different colors in a single metaphase, and the screening of the entire genome for aberrations, has recently become possible through the introduction of multifluor FISH ***(14)*** and spectral karyotyping (SKY) ***(15)***. The value of SKY for improved karyotyping of malignant cells has already been amply demonstrated ***(16–24)***. It is conceivable that the application of SKY as a screening tool in the analysis of hematological malignancies will contribute to the characterization of different disease entities, to the definition of subgroups, and to improvements in diagnosis, prognosis and, potentially, treatment.

1.2. SKY Methodology

SKY is a recently introduced molecular cytogenetic technique that combines combinatorial probe labeling, chromosome painting, fluorescence microscopy, spectroscopy, and charged-coupled device (CCD)-imaging ***(15,25)***.

Twenty-four differentially labeled chromosome-specific painting probes are hybridized simultaneously onto metaphase chromosomes. The basic procedures of pretreatment, hybridization, and detection follow routine FISH protocols. Image acquisition is performed using the SpectraCube™ (ASI), a combination of a Sagnac-Interferometer and a charged-coupled device (CCD) camera mounted to an epifluorescence microscope. A single exposure is sufficient to measure the complete emission spectrum at all image points. Chromosome classification is based on spectral information rather than fluorescence intensities, making SKY a robust technology. Each chromosome is characterized by a unique spectral signature; therefore, subtle translocations, complex rearrangements, and marker chromosomes can be readily identified. The smallest translocation disclosed thus far was approximately 1.5 Mb in size ***(15)***. SKY is less sensitive regarding the detection of intrachromosomal aberrations that do not result in a color change of the aberrant chromosomes, e.g., small deletions, duplications, and inversions. Therefore, the combination of SKY with chromosome banding [(diaminophenylindole, DAPI] or G-banding) is required for a comprehensive analysis of chromosomal rearrangements.

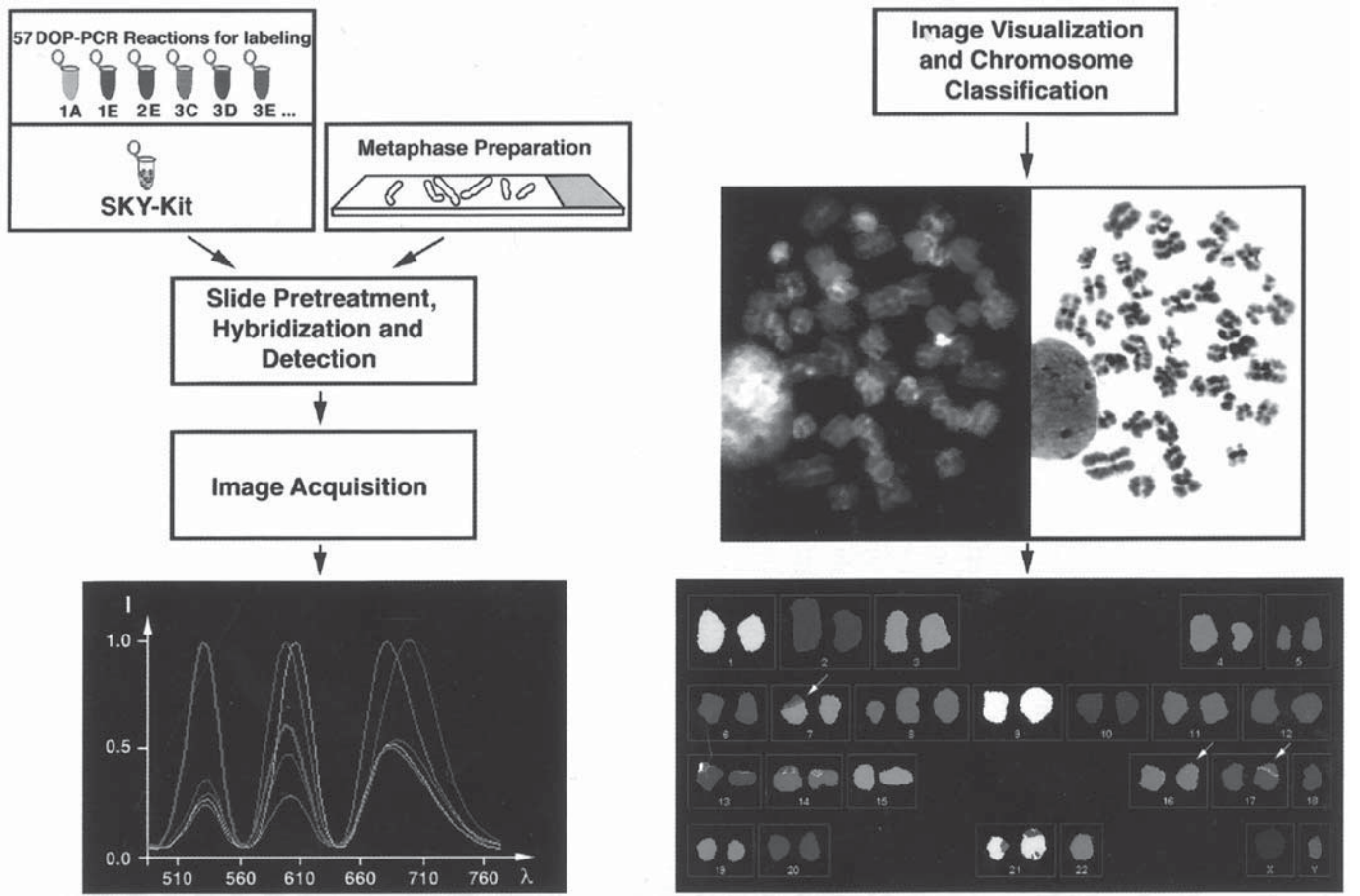
Figure 1 illustrates the different steps of a SKY analysis of a case of AML.

1.3. Application of SKY in Hematological Malignancies

In our own experience, 35% of the aberrations identified in 30 hematological cases with complex karyotypes were newly described by SKY analysis ***(16)***.

Based on the comparison between G-banding and SKY results we detected a high number of new translocations and several normal-appearing chromosomes that were, in fact, structural aberrations, such as translocations. Many complex rearrangements, marker chromosomes, homogeneously staining regions (hsr), as well as double minute chromosomes (dmin) were readily characterized and fully defined. Several numerical aberrations, especially chromosome losses revealed by G-banding were identified as hidden structural aberrations. Interestingly, 90% of all aberrations detected by SKY were structural aberrations. Furthermore, chromosomal breakpoints could be refined by SKY analysis ***(16)***.

The potential of SKY for interpretation of complex karyotypes and detection of new recurrent translocations was further demonstrated in recent analyses of multiple myelomas ***(18,19)***, as well as the newly established megakaryoblastic cell line UoC-M1 ***(20)*** and the cell line HL-60 ***(23)***, as well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1A 1E 2E 3C 3D 3E ...
SKY-Kit
Metaphase Preparation
Slide Pretreatment, Hybridization and Detection
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as cell lines derived from solid tumors: HeLa ***(21)***, BK-10 ***(24)***, and pancreatic cell lines ***(22)***.

As SKY continues to disclose novel chromosomal translocations, it is likely that the analysis of larger series of hematological malignancies will reveal clinically significant translocations and recurring breakpoints. This, in turn, may lead to a better characterization of disease entities or subgroups, improvement of diagnosis, treatment stratification, and ultimately prognosis. Therefore, the utilization of SKY as a screening tool to detect chromosome aberrations in hematological malignancies is one of the most promising applications of this new technique.

New developments like the Cancer Chromosome Aberration Project (C-cap; www.ncbi.nlm.nih.gov/CCAP), which integrates the physical and genetic maps of the human genome with the cytogenetic map, will greatly facilitate the identification of genes involved in chromosomal translocations. C-cap will provide bacterial artificial chromosomes (BAC clones) that cover the human genome with a resolution of 1–2 Mb. The high resolution mapping of chromosomal breakpoints will greatly facilitate the cloning of breakpoint-associated genes.

2. Materials

2.1. SKY Kit Preparation

1. Primer: Telenius 6 MW [5′-CCGACTCGAGNNNNNNATGTGG-3′].
2. Nucleotides for DNA amplification (primary and secondary DOP-PCR): dNTPs 100 m*M* (stock solution: 2 m*M*) (Boehringer Mannheim, Indianapolis, IN).
3. Nucleotides for labeling chromosome paints:
   a. Spectrum Orange dUTP (Vysis, Downers Grove, IL)
   b. Texas Red dUTP (Molecular Probes, Eugene, OR)
   c. Rhodamine 110 dUTP (Perkin Elmer, Foster City, CA)

Fig. 1. *(previous page)* The different steps of the SKY analysis of metaphase chromosomes of an AML case. First, a SKY kit is prepared by combining PCR products from 57 individual labeling reactions and hybridized onto metaphase chromosomes. Slide pretreatment, hybridization, and detection follow standard FISH protocols. Image acquisition is performed using the SpectraCube™, a combination of an interferometer and a CCD camera connected to an epifluorescence microscope. The complete emission spectrum is measured at each image point in a single exposure. The emission spectra of the five single dyes used for labeling are depicted here. After image acquisition, the spectral image is displayed in RGB colors (metaphase spread on the left side) along with the corresponding DAPI-banded chromosomes. Analysis of the spectral information for each image point with the SkyView™ software leads to chromosome classification. The results are then arranged as a karyotype. In this case, three translocations (pointed out by the white arrows) are easily visible.

d. Biotin 16 dUTP (Boehringer Mannheim, Indianapolis, IN)
e. Digoxigenin-11-dUTP (Boehringer Mannheim)
4. For labeling prepare a stock solution of dNTPs with a final concentration of dATP, dCTP, and dGTP of 2 m*M*, but only 1.5 m*M* dTTP.
5. Polymerase: native *Taq* (5 U/µL) (Perkin Elmer)
6. Buffer: 10x PCR Buffer II (Perkin Elmer)

2.2. Precipitation of SKY Kits

1. Human Cot-1-DNA (1 mg/mL) (Life Technologies, BRL, Grand Island, NY).
2. Salmon sperm DNA (9.7 mg/mL) (Sigma, St. Louis, MO).
3. 3 *M* Na-acetate.
4. Deionized formamide (pH 7.5).
5. Mastermix: 20% dextran sulfate in 2X standard saline citrate (SSC; NaCl/Na citrate solution), pH 7.0, autoclave and store aliquots at –20°C

2.3. Slide Pretreatment

1. 2X SSC.
2. RNAse A (Boehringer Mannheim; stock solution: 20 mg/mL).
3. Pepsin (Sigma; stock solution: 100 mg/mL).
4. 0.01 *N* HCl.
5. 1X Phosphate-buffered saline (PBS).
6. PBS/$MgCl_2$: 50 mL 1 *M* $MgCl_2$ + 950 mL 1X PBS.
7. Formaldehyde in 1X PBS/$MgCl_2$: add 2.7 mL of 37% formaldehyde to 100 mL 1X PBS/$MgCl_2$.
8. Ethanol 70, 90, and 100%.

2.4. Denaturation and Hybridization

1. Formamide/2X SSC, per slide: 70 µL deionized FA, 3 µL 20X SSC, 27 µL sterile water, pH 7–7.5.

2.5. Detection

1. Formamide/SSC: 30 mL 20X SSC, 120 mL dH_2O, 150 mL formamide, adjust to pH 7–7.5.
2. 1X SSC.
3. 4X SSC/Tween-20.
4. Blocking solution: 3% Bovine serum albumin (BSA; Boehringer Mannheim). Add 0.3 g BSA to 10 mL 4X SSC/Tween-20, prewarmed to 37°C; store at 4°C.
5. 1% BSA (Boehringer Mannheim). Add 0.1 g BSA to 10 mL 4X SSC/Tween-20.
6. DAPI: 80 ng/mL in 2X SSC; stock solution: 2 mg DAPI/10 mL sterile water.
7. Antifade: Dissolve 100 mg 1,4-phenylenediamine in 2 mL 1X PBS, adjust pH with carbonate buffer to pH 8.0, add 1X PBS to 10 mL, mix with 90 mL glycerol 86%, aliquot, and store at –20°C, protect from light during use.

8. Mouse anti-digoxin (Sigma)
9. Fluorolink-Cy5-avidin (Amersham Life Science Inc., Pittsburgh, PA)
10. Fluorolink-Cy5.5-sheep-anti-mouse-IgG (Amersham Life Science Inc.)

2.6. Equipment

1. PCR-Cycler, PTC-100 (MJ Research Inc., Watertown, MA)
2. Epifluorescence microscope equipped with DAPI-filter TR1, SKY filter V 3.0 (Chroma Technology Corp., Brattleboro, VT)
3. 150 W Xenon lamp (Opti-Quip, Highland Mills, NY)
4. SpectraCube™, Spectral Imaging Acquisition Software and SkyView™ software (Applied Spectral Imaging Ltd., Migdal Ha'Emek, Israel)

3. Methods

3.1. Preparation of Metaphase Chromosomes

The preparation of metaphase chromosomes follows standard cytogenetic protocols *(27)*. Prepared slides should be dehydrated through an ethanol series (70, 90, and 100%, 3 min each) and can be stored in an airtight container with desiccant at –20°C or –80°C. However, best results are normally obtained from slides aged 1 wk stored at room temperature or in a drying oven at 37°C.

3.2. Preparation of SKY Kits

3.2.1. Primary DOP-PCR

Chromosome-specific DNA libraries obtained by bivariate flow sorting are amplified by polymerase chain reaction (PCR) using a degenerate oligonucleotide primer (DOP) *(26)*. As the DNA amplification is sequence nonspecific, using sterile techniques to avoid contamination with genomic DNA is extremely important.

In a second DOP-PCR step, the primary PCR product is labeled for quality control purposes with one fluorescent dye. Each painting probe (for each chromosome) is then hybridized individually onto normal control slides, where only one pair of homologue chromosomes should show specific hybridization signals. The primary PCR-products can only be used for the following steps if no crosshybridization is present and the overall background is low (*see* **Note 1**).

3.2.2. Secondary DOP-PCR

To further amplify the primary PCR products, a second DOP-PCR is performed. This step can be carried out on a regular bench, but again, extreme precautions should be taken to avoid contamination.

1. PCR reaction mix:

Components	µl
DNA (150–200 ng)	2
PCR buffer II (10X)	10
$MgCl_2$ (25 m*M*)	8
dNTP (2 m*M*)	10
dH_2O	65
Primer (100 m*M*)	4
Taq polymerase (5 U/µL)	1
Total volume	100

2. DOP-PCR Program:

Step	Temp. (°C)	Min
1	94	1
2	56	1
3	72	3 with addition of 1 s/cycle
4	Repeat **steps 1–3**, 29 times	
5	72	10
6	4	∞

3. Two microliters of the PCR-product should be run on an 1% agarose gel as a quality control. An intense smear should be visible at about 500 bp–2 kb.
4. Store DNA at –20°C.

3.2.3. Labeling PCR

In this step, fluorescently-labeled nucleotides (dUTP) are incorporated into the previously amplified libraries via DOP-PCR. In order to achieve 24 differentially labeled painting probes, 5 different fluorochromes and their combinations are used. **Table 1** shows our current labeling scheme, which was developed in order to achieve maximal color differences between chromosomes. This scheme leads to 57 PCR-labeling reactions.

1. According to the scheme, label 57 autoclaved PCR tubes.
2. PCR reaction mix:

Components	µL
DNA (400–600 ng)	4
PCR buffer II (10X)	10
$MgCl_2$ (25 m*M*)	8

dNTP (2 m*M*), dTTP (1.5 m*M*)	5
dH_20	65 (for direct) / 67 (for Biotin [BIO] and digoxigenin [DIG])
Primer (100 m*M*)	2
Taq polymerase (5 U/mL)	1
x-dUTP:	
Rhodamine 110, dilute 1:10–0.1 m*M*	5
Spectrum Orange, dilute 1:5–0.2 m*M*	5
Texas Red, dilute 1:5–0.2 m*M*	5
BIO as is 1 m*M*	3
DIG as is 1 m*M*	3
Total volume	100

Table 1
SKY Labeling Scheme

Chromosome	Rhodamine 110	Spectrum Orange	Texas Red	Biotin (Cy 5)	Digoxigenin (Cy 5.5)
1		x			x
2					x
3	x			x	x
4			x	x	
5	x	x	x		x
6			x		x
7	x			x	
8	x				
9	x	x			x
10				x	x
11		x			
12	x		x	x	x
13	x	x			
14			x		
15		x	x	x	
16	x		x	x	
17				x	
18	x	x	x		
19		x		x	
20	x	x		x	
21	x				x
22		x	x	x	x
X	x		x		
Y	x	x		x	x

3. PCR program:

Step	temp. (°C)	minutes
1	94	1
2	56	1
3	72	3 with addition of 1 s/cycle
4	Repeat steps 1–3, 29 times	
5	72	10
6	4	∞

4. Again, run an 1% agarose gel as a quality control. An intense smear should be visible at about 500 bp–2 kb.
5. Prepare one SKY kit (*see* **Subheading 3.2.4.**) and hybridize onto a normal control slide to judge the quality of the kit (*see* **Note 2**).
6. Precipitate all SKY kits after successful test hybridization and store at –20°C.

3.2.4. DNA Precipitation

1. For one SKY kit pipet 4 µL of each chromosome painting probe (400–600 ng), 20 µL human Cot-1 DNA and 1 µL salmon sperm DNA into an Eppendorf tube.
2. Add 1/10 vol 3M Na-acetate and 2.5–3.0 × total volume of cold 100% ethanol.
3. Vortex and precipitate at –20°C overnight or at –80° C for at least 30 min.
4. Centrifuge the precipitated DNA at 4°C, 16,000*g* for 30 min.
5. Remove the supernatant and dry the DNA pellet in a SpeedVac for 5–10 min.
6. Add 6 µL deionized formamide (pH 7.5) and incubate at 37°C for at least 30 min until the pellet is completely dissolved.
7. Add 6 µL Master Mix, vortex, and spin briefly.
8. Kits can be stored at –20°C until used for hybridization.

3.3. Pretreating Slides for SKY

1. Equilibrate slides in 2X SSC at room temperature.
2. Dilute the RNase stock 1:200 in 2X SSC, apply 100–120 µL per slide and cover with a 24 × 60 mm^2 coverslip, incubate at 37°C for 60 min.
3. Prepare 100 ml 0.01 N HCl, adjust to pH 2.0 and pre-warm at 37°C.
4. Remove coverslips and wash three times for 5 min in 2X SSC at room temperature, shaking.
5. Pepsin treatment: Add 30 µL pepsin to a coplin jar, then add 100 mL prewarmed HCl and incubate slides at 37°C for 2 min. (Adjust concentration and time according to amount of cytoplasm. *See* **Note 3**.)
6. Wash twice for 5 min in 1X PBS at room temperature, shaking.
7. Wash once for 5 min in 1X PBS/$MgCl_2$.
8. For postfixation incubate slides for 10 min at room temperature in 1% formaldehyde in 1X PBS/$MgCl_2$.

9. Wash once for 5 min in 1X PBS at room temperature, shaking.
10. Dehydrate slides in 70, 90, and 100% ethanol for 3 min each and air-dry slides.

3.4. Denaturation and Hybridization

3.4.1. Denaturation of the SKY Kit

1. If the SKY kits have been stored at –20°C thaw and prewarm at 37°C for 30 min.
2. Denature SKY kit at 80°C for 5 min in thermomixer or water bath.
3. Allow to preanneal at 37°C for 1–2 h before applying to the slide.

3.4.2. Slide Denaturation

1. Apply 120 µL of 70% formamide/2X SSC to a $24 \times 60 mm^2$ coverslip and touch slide to coverslip.
2. Denature slides at 75°C on a slide warmer for 1.5 min (time may vary depending on the age of the slides and if the slides have been previously G-banded; *see* **Note 4**).
3. Shake the coverslips and immediately place slides in freshly prepared ice-cold 70% ethanol for 3 min, followed by 3 min in 90% and 100% ethanol each.
4. Let slides air-dry.

3.4.3. Hybridization

1. After preannealing add the SKY kit to the preselected hybridization area on the denatured slides and cover with an 18 mm^2 or 22 mm^2 coverslip.
2. Seal coverslips with rubber cement and incubate in a hybridization chamber at 37°C for 48 h (*see* **Note 4**).

3.5. Detection

1. Prewarm solutions (formamide/SSC, 1X SSC, 4X SSC/Tween-20) at 45°C for 30 min before starting the detection (*see* **Note 5**).
2. After the incubation time for hybridization remove rubber cement and coverslips from hybridized slides and dip slides in formamide/SSC until the coverslips slide off.
3. Perform all steps in light-protected coplin jars.
4. Wash slides three times for 5 minutes in formamide/SSC, shaking.
5. Wash slides three times for 5 minutes in 1X SSC, shaking.
6. Dip slides in 4X SSC/Tween-20; do not let them dry.
7. Add 120 µL of blocking solution to 24×60 mm^2 coverslips, touch slides to coverslips, and incubate in hybridization chamber at 37°C for 30 min–1 h.
8. Spin all fluorescent dyes for 3 min at 16,000*g*.
9. Dilute antibodies 1:200 in 1% BSA.
10. Dip slides in 4X SSC/Tween-20; do not let them dry. Add 120 µL of antibody solution containing mouse-anti-digoxin per 24×60 mm^2 coverslip, touch slide to coverslip and incubate in hybridization chamber for one hour at 37°C.
11. Wash slides three times for 5 min in 4 x SSC/Tween-20, shaking.

12. Add 120 µL of antibody solution containing avidin-Cy5 and Cy5.5 anti-mouse per coverslip (24×60 mm^2), touch slide to coverslip, and incubate in hybridization chamber for 1 h at 37°C.
13. Wash slides three times for 5 min in 4X SSC/Tween-20, shaking.
14. Stain with DAPI for 5 min in a light-protected coplin jar.
15. Wash 5 min in sterile H_2O (2X SSC), shaking.
16. Dehydrate slides in an ethanol series (70, 90, and 100%) for 3 min each.
17. Let the slides air-dry and apply 30 µL antifade, cover with 24×60 mm^2 coverslips and store in the dark at 4°C.

3.6. Image Acquisition and Analysis

Image acquisition can be started immediately after detection (*see* **Note 6**). For each metaphase a spectral image is acquired using the specially designed SKY filter (Chroma, Brattleboro, VT) and the SpectraCube™, a combination of a Sagnac-Interferometer and a CCD camera, connected to an epifluorescence microscope. The technical details are described in Schröck et al. ***(15)*** and Garini et al. ***(25)***. The corresponding DAPI image, which leads to a chromosomal banding pattern comparable to the one achieved by G-banding, is acquired using the TR1-filter (Chroma). Heat protection filters should be placed into the light pass, but can be removed if the intensity of the fluorescent dyes with emission in the far red range is weak.

After image acquisition, the spectral image is first displayed in the RGB (red-green-blue) colors, which permits an evaluation of the quality of the hybridization (*see* **Fig. 1**). When the SkyView™ software is used, both the spectral and the DAPI image are then analyzed simultaneously. By correlating the spectral information with the labeling scheme and the reference spectra of the five fluorescent dyes stored in a so-called "combinatorial table" (ctb) file, a specific pseudocolor is assigned to each image point. Therefore, all material belonging to the same chromosome will be displayed in the same pseudocolor and chromosomal aberrations will be easily visible. The results of the SKY classification are automatically arranged in a karyotypic fashion (*see* **Fig. 1**).

4. Notes

1. Primary DOP-PCR: Test hybridization of the flow-sorted chromosomes for quality control is crucial. They can be used if the control hybridization shows painting of the respective pair of homologue chromosomes only and the overall background is low.
2. Labeling DOP-PCR:
   a. One SKY kit should be precipitated according to the protocol and hybridized onto normal metaphases as quality control for the batch of kits produced.
   b. With this test hybridization the overall homogeneity of the painting pattern and the suppression of heterochromatin should be judged.

   c. The signal-to-noise ratio should be evaluated. When the software for image acquisition is used, the highest and lowest values for the fluorescence intensity within the image are displayed. A difference of at least 100 counts between the intensity along chromosomes and background must be achieved.
   d. In the RGB image, good color separation between chromosomes displayed in red, green, or blue must be present.
   e. The spectra of the single dyes of this test hybridization should be compared to and should be similar to the reference spectra stored in the ctb file.
   f. If the SKY kit is of good quality, the automated classification with the SkyView™ software of a normal metaphase should be correct.
3. Pretreating slides with pepsin to remove residual cytoplasm is a crucial step, as overtreatment with pepsin leads to reduced signal intensity and impaired chromosome morphology, which therefore compromises SKY results. Pepsin concentration and time must therefore be adjusted according to the amount of cytoplasm. Cytoplasm is visible as opaque material around the metaphase chromosomes. If no cytoplasm is present pepsin treatment may not be necessary at all.
4. Denaturation and hybridization: Denaturation of slides can also be performed by preheating 70% formamide/2X SSC in a coplin jar in a water bath to 72°C. Avoid drying out of the SKY kit during hybridization.
5. Detection: Preheat all solutions to 45°C prior to the detection process. During detection avoid exposure to light as much as possible and avoid air-drying the slides in between the different steps.
6. Image acquisition and analysis: Acquire images within a week after detecting SKY hybridization.

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2

Polymerase Chain Reaction

5

Molecular Genetic Methods in Diagnosis and Treatment

Pamela Ward and Alan Kinniburgh

1. Introduction

1.1. Overview

During the past 20 yr, molecular biology and molecular genetics have provided techniques and procedures that allow the precise diagnoses of leukemias and lymphomas (for a review, *see* **ref.** ***1***). These methodologies have provided powerful, robust, and precise characterizations of genetic mutations and gene rearrangements (both normal and abnormal) that have been applied to the diagnosis and treatment of these malignancies. In this chapter we discuss methodologies currently utilized within the molecular diagnostic laboratory of cancer centers and major academic hospitals. We introduce these methodologies in both general terms and within the context of a differential diagnosis of leukemia/lymphoma. The long-term goal of the molecular hematologist/oncologist is to describe all mutations that occur within a given type or subtype of leukemia/lymphoma. This involves the discovery of new somatic and possibly hereditary mutations that contribute to the genesis of these hematologic malignancies. However, for this information to be useful in treatment regimens, therapies that target or affect these growth-dysregulating mutations must be found. Because diagnosis of mutation and gene rearrangement in the hematologic malignancies is far ahead of therapeutic modalities, we also discuss new research tools that will have impact on the experimental hematologist.

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1.2. Assays Used in Diagnosing Leukemia

1.2.1. Southern Blotting/Hybridization Using IgH and TCR β Probes for Lineage Determination in Lymphoma and Lymphocytic Leukemias

The enormous diversity of the antigen-specific receptors of lymphocytes is generated by the recombination of variable (V), diversity (D), and joining (J) region gene segments to a constant (C) region segment. During differentiation, an appropriate set of V(D)J and C segments is spliced together to generate a complete gene which is transcribed and translated into either a T-cell receptor (TCR) or an immunoglobulin (Ig) chain ***(2,3)***. Lymphomas and lymphocytic leukemias result from the clonal expansion of a single transformed T- or B-lineage cell. The transformed cell displays its own unique rearrangement of the V(D)J and C region genes that is immutable and replicated during clonal expansion.

The vast majority of the T- and B-genotyping assays are performed using the Southern blotting technique, in which DNA is extracted from putative leukemic or lymphomic tissues and digested with restriction endonucleases. DNA fragments are then separated by electrophoresis through an agarose gel, and the DNA is blotted onto a nylon membrane and hybridized with radiolabeled cloned probes ***(1,4,5)***. A Southern blot assay will identify clonal Ig and TCR gene rearrangements and is useful in several different ways. First, the assay can rule out polyclonal, hyperplastic cell populations. In cases of leukocytosis or lymphadenopathy, standard histopathology may be ambiguous, and detection of a clonal TCR and/or Ig gene rearrangement may be helpful. Second, there may be resolution of the B- or T-cell origin of the disease. In fact, in rare instances biphenotypic disease, marked by both TCR and Ig gene rearrangements, may be detected. Of course, standard histopathologic and flow-cytometric tests complete the repertoire of diagnostic tools; all tests are independently predictive. The most informative B-genotyping assays analyze the IgH chain using a joining region (JH) genomic probe with the restriction digests of *Hind*III, *Eco*RI, and *Bam*HI (*see* **Fig. 1**). This is predicated by the sequence of genetic events that occurs during B-cell differentiation. First, the Ig molecule is comprised of heavy (H) and light (L) chains, of which the IgH genes are the first to rearrange, followed by the IgL kappa and lambda chains ***(6)***. Second, D-to-J precedes V-to-DJ gene segment joining. Thus, regardless of the incomplete status of the VDJ recombination, a JH probe will detect all B-cell lineage clonal gene rearrangements. The exception will be in the case of pro-B cell acute lymphocytic leukemia (ALL) malignancies, in which the malignant transformation occurred while the IgH gene segments were in a germline configuration.

During T-cell differentiation, greater than 85% of mature T-cells express the α/β classic TCR cell surface heterodimer, whereas the remainder express

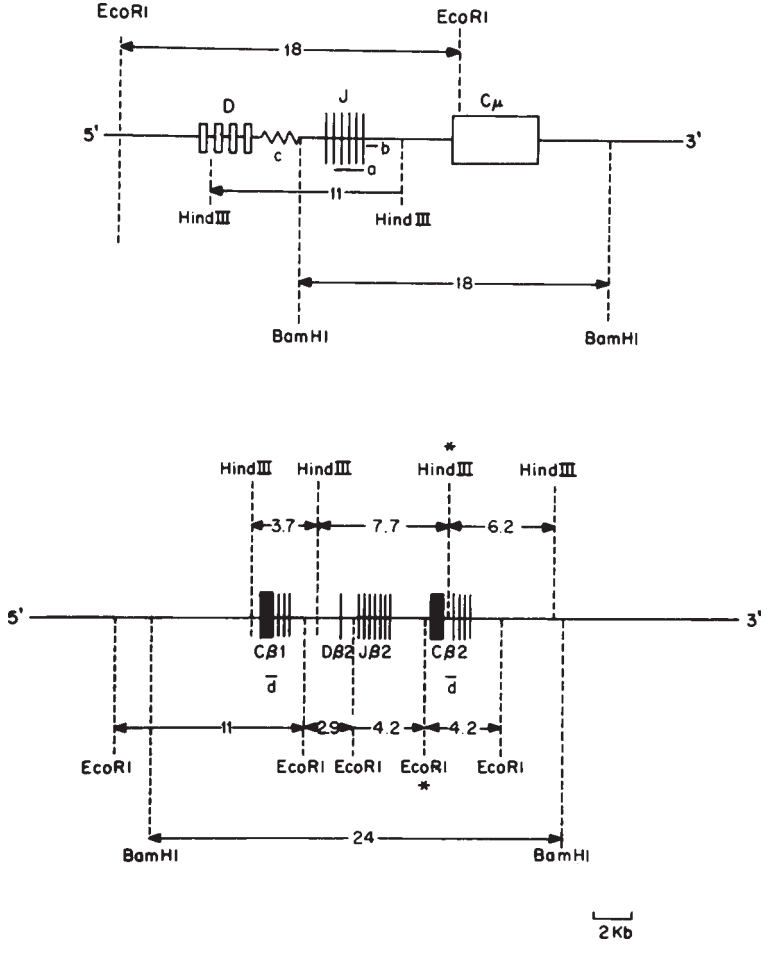


Fig. 1. Restriction endonuclease maps of the human IgH and TCR-β genes. **Upper:** Organization of the diversity (D), joining (J), and constant (Cμ) regions of IgH relative to the location of the *Sau*3a J_H DNA probe **(a)**, the $IgHJ_6$ DNA probe **(b)**, and the hypervariable polymorphism region 5′ to the J_H region **(c)**. **Lower:** Organization of the constant regions (Cβ1 and Cβ2), diversity (Dβ2) and joining (Jβ2) regions of the TCR-β relative to the location of the C_Tβ probe, which hybridizes to both Cβ1 and Cβ2 regions. *The partially resistant *Eco*RI and *Hind*III sites.

the γ/δ heterodimer. Initially, the TCR-δ chain begins to rearrange, followed by the TCR-γ chain. The successful rearrangement of both these chains leads to the expression of a functional γ/δ TCR. However, a successful TCR-β chain rearrangement often interrupts the γ/δ rearrangement and leads to the expression of an α/β TCR molecule. In the T-genotyping assay, virtually all TCR

gene rearrangements are detected using the $C_T\beta$ probe, which recognizes both constant regions using the restriction digests of *Hind*III, *Eco*RI, and *Bam*HI (*see* **Fig. 1**). The TCR-Jγ probe will be informative in cases in which clonal TCR-β are not detected, yet other pathologic tests indicate T-cell disease.

1.2.2. Reverse Transcription Polymerase Chain Reaction (RT-PCR) Using RNA Oncogene Translocations within Transcribed Regions

Reverse transcription of polyadenylated RNA (the vast majority of cellular mRNA) with the subsequent amplification of the copied DNA via the polymerase chain reaction is an important technique in assaying so-called "fusion" transcripts. Fusion transcripts occur in rare instances in which chromosomal translocation fuses transcribed regions of two genes. These may result in fusion proteins in which coding information from one gene is fused to the coding information of another gene, producing a hybrid protein. These proteins may retain functionality, as in the case of the bcr/abl fusion protein, but the regulation of these proteins' functions may be abnormal. Chronic myelogenous leukemia (CML) is typified by the presence of the Philadelphia chromosome (Ph1), which is found in >93% of all cases. The Ph1 translocation t(9;22) (q34;q11) fuses the *bcr* gene to the *abl* gene resulting in the production of a composite or fusion mRNA and protein ***(7)***. This hybrid protein coaxes several extra cell divisions from myeloid progenitor cells without affecting differentiation, at least in the chronic phase of the disease. At some point in the disease course, patients suffer a blastic crisis, in which malignant, immature cells proliferate extensively. Because the majority of patients present with abnormal numbers of histopathologically, normal cells, molecular and cytogenetic conformation of the *bcr/abl* fusion gene is the cornerstone of molecular diagnosis ***(8)***. The molecular analysis of the t(9;22) is performed by a RT-PCR assay using primers that span the two breakpoint cluster regions within the translocations. Because this procedure uses the fusion mRNA as the substrate for analysis, this assay is highly sensitive. In controlled, reconstructed experiments with normal and chronic phase sample RNAs, sensitivities of one malignant cell in 100,000 to 1,000,000 can be achieved ***(8)***. The sensitivity is high compared to standard PCR because there are many copies of mRNA per cell, whereas each cell contains only one copy of the translocated fusion gene.

1.2.3. PCR Using DNA for the Detection of Oncogene translocations That Occur Outside of Transcribed Regions

The most frequent genetic abnormality in non-Hodgkin's lymphoma is a translocation of a segment of chromosome 18 to chromosome 14 [t(14;18)] ***(9–11)***; other translocations observed in non-Hodgkin's lymphoma include t(8;14), t(8;11), t(11;14), and t(2;5). Specifically, the t(14;18) occurs in >85%

of follicular lymphomas, which constitute the majority of non-Hodgkin's lymphomas.

The t(14;18) translocation results in the rearrangement of the *bcl-2* gene, which resides on chromosome 18 (band q21). In the majority of cases, the molecular breakpoints on chromosome 18q21 lie within a 2.8-kilobase (kb) major breakpoint region (MBR) in the 3′ untranslated region of the gene; most breakpoints in the MBR cluster within a 150-base pair (bp) region. A second cluster of 18q21 breakpoints occurs approximately 20 kb downstream of MBR in a 500-bp region called the minor cluster region (mcr). In all cases, the *bcl-2* gene translocates into one of the six joining (J_H) segments of the immunoglobulin heavy chain gene (IgH) of chromosome 14 (band q32). The translocation creates a unique segment of DNA in the lymphoma cells composed of a joined *bcl-2*/J_H sequence.

The unique *bcl-2*/J_H DNA sequence is exploited as a cancer-specific marker for cells containing the t(14;18) translocation by amplification of the DNA sequence at the *bcl-2*/J_H junction. Oligonucleotide primers specific for the regions of chromosomes 14 and 18 flanking the translocation are used ***(12–14)***. The standard t(14;18) diagnostic assay utilizes a nested primer amplification reaction, a type of polymerase chain reaction (PCR) that is specific for either the MBR or the mcr, as described by Gribben and associates ***(15)***. The assay will detect t(14;18)-containing cells for either breakpoint region among a normal background and will discriminate all t(14;18) translocations that occur within these regions.

The t(14;18) assay may be used in several ways. It can be used first to confirm the initial diagnosis of follicular lymphoma. It can also be used second during treatment to monitor the presence of minimal residual disease (MRD). In these follow-up studies, samples obtained from bone-core biopsies and marrow aspirates are more sensitive than those from peripheral blood in detection of MRD ***(16)***.

Although the PCR detection assay is very sensitive, it is also very specific. Because the MBR spans a 2.8-kb segment of the gene, breakpoints occurring at the 5′ end of the 150-bp region will not be detected ***(17,18)***. If the cytogenetics of the tumor in question is not informative, the possibility of a t(14;18) assay false-negative result can be circumvented by use of the Southern blot analysis. A Southern blot analysis is less sensitive than a PCR-based assay (5– to 10% vs 0.001%), but it is more inclusive so that a 2.8-kb MBR DNA probe should detect breakpoints across the entire MBR when more than 5% of the cells in the sample contain the t(14;18) translocation ***(12,17)***. After a positive identification of a breakpoint within the MBR, secondary PCR reactions can be performed with a series of alternative 5′ primers, which incrementally move further upstream through the MBR.

The utility of the t(14;18) translocation as a prognostic indicator is controversial. On one hand, this translocation is present in the majority of indolent low- to intermediate-grade lymphomas that require no treatment for years but that ultimately can cause relapse. The translocation is not generally associated with the aggressive high-grade lymphomas in which a considerable degree of chemotherapeutic success can be achieved. On the other hand, the minority of low- to intermediate-grade lymphomas that do not carry the t(14;18) translocation appear to follow the same clinical course as those with the translocation ***(19)***. However, irrespective of whether the t(14;18) translocation has any prognostic significance, it provides a cancer-specific marker to follow MRD. Studies show the persistence of PCR-detectable t(14;18)-bearing cells in both postautologous bone marrow transplantation patients and in those in complete clinical remission. This status may be maintained for years, but the presence of these cells is ultimately associated with an increased incidence of relapse ***(20)***.

B-cell chronic lymphocytic leukemia (CLL) is the most prevalent adult leukemia in the Western hemisphere. It presents as an accumulation of circulating malignant lymphocytes in the G_0 phase of the cell cycle. As with the follicular lymphomas, B-cell CLLs can follow an indolent course in which the malignant cells constitute a nonproliferative pool and remain elusive. Furthermore, most cases of CLL overexpress the bcl-2 protein, which presumably confers prolonged survival on the cell. In the majority of CLL cases, the mechanisms for this overexpression are not clearly understood; they may include point mutations in the promoter region, transregulatory mechanisms, or posttranslation events ***(21–23)***.

Approximately 10% of CLL cells show variant *bcl-2* translocations involving either the kappa or lambda immunoglobulin chains. In both t(2;18) and t(18;22) translocations, the *bcl-2* gene rearrangements involve the 5′ region of the gene upstream of the open reading frame; in the light chains, the breakpoints occur within the J gene segments ***(23)***. At this time, these translocations are not routinely exploited diagnostically either to confirm a diagnosis or to follow MRD. However, detection of the classic t(14;18) translocation is used to rule out the possibility of CLL and to confirm that the malignancy is a low-grade lymphoma.

1.2.4. Other Translocation Breakpoints

The aforementioned diagnostic assays illustrate the fundamental distinction of detecting translocations within transcribed vs nontranscribed regions, in which the methodology of the test exploits the biology of the targeted chromosomal rearrangement. The number of translocations associated with the hematologic malignancies is extensive ***(25,26)***. However, all translocations fall into one of three categories, a few of which are listed in **Table 1**. Those transloca-

Table 1
Translocations in Hematologic Malignancies Divided Into Three Groups: I, Oncogenes Juxtaposed to Ig loci; II, Oncogenes Juxtaposed to TCR Loci; and III, Fusion Oncoproteins

Group	Malignancy	Rearrangement	Gene(s)
I	Burkitts lymphoma	t(8;14)	*myc*
	B-CLL	t(11;14)	*bcl-1*
	B-CLL	t(14;19)	*bcl-3*
II	T-ALL	t(8;14)	*myc*
	T-ALL	t(7;19)	*lyl-1*
	T-ALL	t(1;14)	*tcl-5*
	T-ALL	t(11;14)	*rbnt-1*
III	APL	t(15;17)	*pml/rara*
	AML	t(6;9)	*dek/can*

tions in groups I and II involve oncogenes juxtaposed to Ig or TCR loci respectively, in which detection would use DNA-based PCR assays. Translocations of the type illustrated in group III occur within transcribed regions to create fusion oncoproteins, whereby detection would be achieved through RT-PCR-based assays. Thus, the diagnostician need only substitute breakpoint specific primers into either of these two assays in order to achieve detection of any known translocation. However, for a test to have diagnostic and prognostic utility, the targeted breakpoint must be the molecular hallmark of the disease. Furthermore, the malignancy must occur at sufficient frequency in order to warrant initial setup of the molecular test.

1.3. Research Methods Used in Analyzing Leukemia Gene Rearrangements and Gene Deletions

Positional cloning technologies have been exploited in the isolation of genes involved in translocation breakpoints. These technologies involve gene mapping, large DNA fragment cloning vectors, and exon-trapping techniques (for the analysis of expressed DNA sequences, i.e., protein-encoding genes). The methodologies are beyond the scope of this chapter. Suffice it to say that the known translocation breakpoints have the common theme of misexpression of

a cellular protooncogene. The methods described within this chapter are an integral part of these methodologies. For example, any known translocation breakpoints can be assayed by Southern blotting/hybridization techniques given that DNA probes to sequences near an altered restriction endonuclease site are available. The hunt for new tumor suppressor genes that are exclusively or are representatively deleted in leukemias has not borne positive results. Studies examining loss of heterozygosity (LOH) in leukemias, the hallmark of tumor suppressor genes, have been performed in hematological malignancies. These studies have utilized PCR of microsatellites as well as standard restriction fragment length polymorphisms (which use Southern blotting/hybridization procedures). For example, Miyake et al. ***(27)***, have shown the loss of heterozygosity in AML (acute myeloid leukemia), ALL, and certain CML leukemia cell samples for a well-characterized tumor suppressor gene, deleted in colorectal cancer (DCC). This implicates the DCC gene in the leukemogenic process. A more general study of LOH in various leukemias using microsatellite PCR methods has shown that 25% of the markers chosen show LOH. The reader is referred to these authors for procedures in adapting the PCR and Southern blotting procedures described here for such searches for tumor suppressor gene inactivation.

1.4. Future Research Techniques for Understanding Genome Expression Changes in Hematologic Malignancies

Recently, there has been a rapid increase in our understanding of expressed DNA sequences. EST (expressed sequence tags) databases have allowed researchers to rapidly scan expressed sequences by partial sequencing. One such technique, termed serial analysis of gene expression (SAGE), has been utilized to examine the types and abundance of all the mRNAs expressed within cancer cells and these have been compared with the mRNA sequences found in cognate normal tissues or cell types ***(28,29)***. Sequences that vary more than 10-fold in expression levels have been compared in colon and pancreatic cancer. Interestingly, only 500 mRNA sequences have been shown to vary. Approximately half are more highly represented in tumors than normal cells and the other half are under represented in tumor cells vs normal. Obviously, not all of these changes are causative of the malignant phenotype. For example, Vogelstein's group has found that ribosomal protein gene's expression is increased, as would be expected for any cell that is growing ***(29)***. Presumably these will appear in cross-tumor comparisons, along with certain common over- or underexpressed oncogenes and tumor suppressor genes. The hematologic malignancies, with relatively stable genomes, should allow a rapid focus on genes that are causative. Of course, preparing cognate "normal" cells that share the same developmental lineage and developmental state as the malignant

hematologic cell will be key in elucidating cancer-specific changes in gene expression.

The ability to examine most of the changes in the expression of "tumor-specific genes" (most because some proteins quantities are regulated via proteosomeal degradation and other posttranscriptional means) will allow drug discovery to focus on discrete targets. Also, when these procedures can be performed in real time, the examination of gene expression changes during therapy in the tumor cells of patients will allow the efficacy to be examined more rapidly than monitoring tumor cell number, morphology, and the like. This new "predictive medicine" may, in the near future, allow a customized therapeutic regimen to be developed for leukemia and lymphoma.

2. Materials

2.1. Evaluation of Sample Sufficiency

Bone marrow aspirates and peripheral blood samples are submitted to the laboratory in EDTA-treated tubes; heparin interferes with the activity of *Taq* polymerase, and such anticoagulants are avoided. Biopsy specimens are minced with curved scissors prior to mechanical dispersion in the tissue douncers. Addition of 2-mercaptoethanol (1 µl/mL DNA isolation buffer) to the mixture augments protein degradation by dissociating disulfide bonds. A minimum volume of 2.5 mL of aspirate and 10 mL of blood is considered sufficient for analysis; obviously, the number of nucleated cells recovered varies according to the white count. A Southern-based test requires 30 µg DNA, l0 µg to be digested with each of three restriction enzymes. A diploid cell contains 6 pg DNA. Thus, theoretically, $5 \times l0^6$ cells are required to recover 30 µg DNA. However, assuming a 50% loss of the material, a minimum of 10^7 cells would be required.

2.2. Handling Tissue Samples

Tissue samples are submitted snap frozen in liquid nitrogen. Alternatively, residual frozen blocks embedded in ornithine carbamyl transferase (OCT) (Miles Laboratories, Elkart, IN) may be accepted. Samples embedded in OCT are rinsed in 1X phosphate-buffered saline (PBS) before dissociating the tissue. Tissue volumes are rarely insufficient. Skin biopsy samples are so variable in terms of lesion:collagenous tissue ratios, however, that complete samples are processed regardless of size.

2.3. Solutions and Reagents

1. Red blood cell (RBC) lysis buffer: 155 m*M* NH_4Cl, 10 m*M* $KHCO_3$, 1 m*M* disodium ethylenediaminetetraacetic acid [EDTA].
2. DNA isolation buffer: 150 m*M* NaCl, 50 m*M* EDTA, pH 8.0, 20 m*M* 2M Tris-HCl, pH 8.0.

3. Phenol : chloroform : isoamyl alcohol (PCIA) (25 : 24 : 1).
4. CIA (chloroform : isoamyl alcohol 24 : 1).
5. TE(8): 10 m*M* Tris-HCl, 1 m*M* disodium EDTA, pH 8.0.
6. RAPID DNA lysis buffer: (8% dodecyltrimethyl ammonium bromide, 1.5 *M* NaCl, 100 m*M* Tris-HCl, 50 m*M* disodium EDTA, pH 8.6.
7. GITC: 4.2 *M* guanidium isocyanate, 25 m*M* Na_3 citrate, 1% 2-mercaptoethanol.
8. 5.8 *M* CsCl (buoyant destiny [ρ] = 1.8g/mL).
9. 1X STE: 100 m*M* NaCl, 10 m*M* Tris-HCl, 1 m*M* disodium EDTA, pH 8.0.
10. 20X standard saline citrate (SSC): 3 *M* NaCl, 0.3 *M* Na_3 citrate, pH 7.0.
11. 0.8% agarose gel (20 cm × 20 cm × 0.6cm) in 1X TBE (90 m*M*, Tris-HCl; 90 m*M* boric acid, 2 m*M* EDTA, pH 8.3).
12. Denaturation solution: 600 m*M* NaCl : 0.2 *N* NaOH.
13. Sheets of GB004 and GB002 gel blot paper (Schleicher and Schuell, Keene, NH).
14. Zetabind (CUNO, Meriden, CT).
15. Hexamers (N6) Sigma (St. Louis, MO).
16. Wash buffer A: 0.5% (W/V) bovine serum albumin (BSA), 1 m*M* EDTA, 5% sodium dodecyl sulfate (SDS), 10 m*M* Na_2HPO_4, l0 m*M* NaH_2PO_4, pH 6.8.
17. Wash buffer B: 1 m*M* EDTA, 1% SDS, 10 m*M* Na_2HPO_4, l0 m*M* NaH_2PO_4, pH 6.8.
18. Hybridization buffer: 1% (w/v) BSA, 0.25 *M* Na_2HPO_4, 0.29 *M* NaH_2PO_4, 1 m*M* EDTA, 7% SDS, pH 7.0.
19. RPRB (Random Primer Reaction Buffer): 0.5 *M* HEPES, pH 6.6, 5 m*M* $MgC1_2$, 125 m*M* Tris-HCl, pH 8.0, 25 m*M* 2-mercaptoethanol, 500 µM dATP,500 µ*M* dGTP, 500 µM TTP.
20. *Hind*III, *Eco*RI, and *Bam*HI restriction endonucleases.
21. Klenow fragment of *Escherichia coli* DNA polymerase I.
22. Stratagene Push columns (La Jolla, CA).

2.4. Equipment

1. Shaking water bath, Sci-Era, Bellco (Vineland, NJ).
2. Orbital shaker, JR Scientific (Rochester, NY).
3. Hematology/chemistry mixer, Fisher Scientific (Pittsburgh, PA).
4. Power supply, Bio-Rad 200/2.0 (Richmond, CA).
5. Horizontal gel electrophoresis apparatus, IBI (New Haven, CT).
6. Ultraviolet (UV) spectrophotometer, Beckman DU-64 (Palo Alto, CA).
7. SpeedVac centrifuge, Savant SCll0A w/ refrigerated trap (Farmingdale, NY).
8. Low-speed centrifuge, Beckman GS-6R w/ GH3.7 rotor.
9. Ultracentrifuge, Beckman TLX-120 w/ TLS-55 rotor.
10. Microfuge, Beckman model 12 w/bowl rotor.
11. Microfuge, Spectrafuge 16M, Labnet (Woodbridge, NJ).
12. Thermacycler, Gene~PCR system 9600, PE Biosystems (Foster City, CA).
13. UV crosslinker, Ultra-Lum UVC-515 (Carson, CA).
14. UV hand-held 254 nm UV lamp, UVP UVGL-25 (San Gabriel, CA).
15. UV transilluminator, Foto/PrepI, Fotodyne (New Berlin, WI).
16. Camera system, Fotodyne MP-4 land camera.

17. Blotting apparatus, Schleicher and Schuell Turboblotter.
18. Push column beta shield device, Stratagene.
19. Hybridization oven, Hybaid MK II (Franklin, MA).
20. Geiger counter, Ludlum model 3 (Sweetwater, TX).
21. Liquid scintillation counter, LKB Wallac 1214 Rackbeta (Gaithersburg, MD) X-ray developer, Kodak M35A X-omat Processor (Rochester, NY).

3. Methods

3.1. Nucleic Acid Preparation

Nucleic acid isolation is a critical first step in the molecular detection of a genetic marker. Yield is an important consideration for samples such as skin biopsies, and the quality of extracted material will dictate the enzymatic action of both restriction endonucleases and polymerases. There is a plethora of commercial kits on the market for the isolation of DNA/RNA. The advantage of using kits is predominantly convenience, which often eliminates the use of organics/ultracentrifuge. However, there are a number of standard isolation protocols that generate high-quality nucleic acids with minimal reagent costs *(33,31)*. The choice of procedure will be determined to some extent by the quality of DNA required. For Southern-based analyses, DNA of the highest quality is essential, so that the DNA is fragmented by the restriction endonucleases alone and not by the isolation procedure itself. For PCR-based assays, it is not essential to preserve the integrity of the DNA. In fact, mechanical fragmentation of high-molecular-weight genomic DNA prior to amplification increases the efficiency of primer annealing and amplification. Prior to nucleic acid isolation, contaminating red blood cells must be removed from the clinical samples. Some commercial kits offer fast extraction methods for PCR-based assays and omit the RBC lysis step. In our experience, this practice yields polypeptide-contaminated DNA, which is subject to amplification failures. Thus, it is our recommendation that the time be taken to perform this step, especially in determining minimal residual disease; for a Southern-based analysis, this step is essential (*see* **Note 1**).

3.1.1. RBC Lysis Step

RBC lysis is also performed on splenic and hemorrhagic tissues. These tissues are mechanically dissociated in the tissue douncers with the RBC lysis buffer before incubation at 4°C. We use the following procedure:

1. Disperse sample in 3 vol of ice-cold RBC lysis buffer.
2. Incubate at 4°C for 15 min.
3. Centrifuge at 1000*g* for 10 min at room temperature.
4. Reincubate pellet with 10 mL RBC lysis buffer, if necessary.

5. Wash final pellet in 10 mL PBS.
6. Centrifuge and resuspend pellet as per isolation protocol.

3.1.2. DNA Isolation for Southern Analyses

1. Disperse cell pellet (2.5×10^6 cells/mL) in DNA isolation buffer using a tissue douncer.
2. Add proteinase K to a final concentration of 100 (g/mL and 20% SDS to final concentration of 1%. Invert to mix.
3. Incubate overnight at 50°C.
4. Add 1 vol of PCIA (25 : 24 : 1). Shake to mix—do not vortex.
5. Spin for 5 min 2000*g* at room temperature.
6. Transfer aqueous phase using Pasteur pipet, with broken tip, to a fresh tube. It is important to avoid proteins that precipitate at the aqueous/organic interface.
7. Repeat PCIA extraction.
8. Add 1 vol CIA (chloroform : isoamyl alcohol 24 : 1). Shake to mix.
9. Spin for 5 min at room temperature.
10. Transfer aqueous phase. Repeat CIA extraction.
11. To aqueous phase add 0.1 vol 2 *M* Na acetate, pH5.5.
12. Add 1 vol 2-propanol at room temperature.
13. Gently invert tube to precipitate DNA.
14. Collect DNA on hooked Pasteur pipet.
15. Wash DNA pellet in ice-cold 70% ethanol to remove excess salt.
16. Resuspend DNA in TE(8) at a final concentration of 0.75–1.00 µg/µL. DNA will dissolve under slightly alkaline conditions.
17. Allow the DNA to dissolve overnight at room temperature in an orbital mixer. It is important to maximally disperse the cell pellet/tissue in the DNA isolation buffer such that cell clumps are not readily visible by the naked eye. This will increase the yield of DNA recovered.

3.1.3. DNA Isolation for PCR-Based Assays

1. Disperse 10^6 cells in 600 µL RAPID DNA lysis buffer.
2. Rotate overnight in an orbital mixer at room temperature, thereby increasing the yield of DNA recovery.
3. Incubate at 68°C for 5 min.
4. Add 600 µL chloroform. Mix by inversion several times.
5. Spin for 2 min at 12,000*g* at room temperature.
6. Transfer aqueous phase to fresh microtube.
7. Repeat **steps 4–6**. If aqueous phase remains contaminated with protein, repeat chloroform extractions.
8. Add 900 µL ddH_2O. Add 100 µL 5% CTABS (5% hexadecylmethyl ammonium bromide, 0.4 *M* NaCl).

9. Mix by inversion several times.
10. Spin for 2 min at room temperature.
11. Decant supernatant. Resuspend pellet in 300 µL 1.2 *M* NaCl.
12. Allow tubes to mix at room temperature overnight.
13. Add 750 µL 100% ethanol. Mix by inversion.
14. Spin at 4°C for 10 min.
15. Remove supernatant. Wash pellet in ice-cold 80% ethanol.
16. Spin at 4°C 10 min.
17. Carefully remove the supernatant. Dry the pellet in the refrigerated centrifuge vacuum dryer (SpeedVac) for 5 min.
18. Resuspend in 100–500 µL ddH_2O, depending on the size of the DNA pellet (final concentration of 100 ng/µL).
19. Place in the orbital mixer to rotate overnight.

3.1.4. RNA Isolation by Density Gradient Centrifugation

1. Disperse cells ($<10^8$ cells/5 mL) in 3 vol GITC (4.2 *M* guanidium isocyanate, 25 m*M* Na_3 citrates, 1% 2-mercaptoethanol). Layer onto 1 vol 5.8 *M* $CsCl_2$ (=1.8 g/mL) cushion.
2. Centrifuge in a TLS-55 or SW60 rotor in a Beckman ultracentrifuge at 110,000*g* for 18 h.
3. Remove supernatant. NB: DNA (buoyant density [ρ] = 1.7 g/mL) will float on the CsCl cushion; be careful not to contaminate RNA (ρ = 1.9 g/mL), which has precipitated to the bottom of the tube.
4. Resuspend RNA pellet in 500 µL 1X STE and Vortex.
5. Add 2 vol ice-cold 100% ethanol. Vortex. Incubate at –20°C for 20 min.
6. Spin at 4°C for 10 min.
7. Decant supernatant. Repeat **steps 4–6**.
8. Decant supernatant. Invert tubes to drain for 5 min. Dry under centrifuge vacuum.
9. Resuspend RNA in 100–500 µL ddH_2O at a concentration of approx 100 ng/µL.

3.2. Nucleic Acid Quantitation by Absorption Spectroscopy

The spectroscopic absorption of the isolated nucleic acid is measured at different wavelengths to assess its purity and concentration. A_{260} measurements are quantitative for relatively pure nucleic acid preparations in microgram quantities. Absorbency readings cannot discriminate between DNA and RNA. The A_{260}/A_{280} ratio is the standard indicator of purity; proteins have a peak absorption at 280 nm that will reduce the 260/280 ratio. Absorbency at 320 nm indicates particulate matter in the solution, whereas contaminants containing peptide bonds absorb at 230 nm, and phenolic contaminants absorb at 270 nm ***(33)***. (*See* **Note 1.a.**)

Indicator ratios	OD readings
A_{260}/A_{280} ~ 1.8	A_{260} units of double stranded DNA = 50 mg/mL
A_{260}/A_{230} ~ 1.8	A_{260} units of single stranded RNA = 40 mg/mL
A_{260}/A_{270} ~ 0.7	
A_{320}/A_{260} ~ 0.05	

3.3. Southern Blotting/Hybridization in Diagnostic Gene Rearrangement Assays

A Southern blot analysis is a multistaged procedure that is divided into five distinct stages, listed as follows. In the clinical laboratory, critical indicators are evaluated to ensure that the sample has been manipulated successfully.

3.3.1. Restriction endonuclease digestion of DNA:

For IgH and TCR-β gene rearrangement analyses, the standard enzymes used are *Hind*III, *Eco*RI, and *Bam*HI (*see* **Fig. 1**). For each analysis to be performed, positive and negative controls must be used. Our laboratory spikes 5% positive control DNA into the negative control reaction tubes, thereby establishing a detection sensitivity for the assay. Each clinical sample and control DNA is digested with all three enzymes. The volume of enzyme used is limited to approx 10% of total volume, otherwise, the glycerol solution in which the enzymes are suspended inhibits their action.

1. Aliquot: 10 µg DNA, 3 µL 10X buffer, 50 U restriction endonuclease, ddH_2O to bring volume to 30 µL (when pipetting high-molecular-weight genomic DNA, it is important to use wide-orifice tips to minimize DNA shearing; also see Note 7).
2. Incubate at 37°C overnight.
3. Add 3.5 µL 10X loading dye. Load immediately onto a gel, or hold at –20°C.

3.3.2. Agarose Gel Electrophoresis

Standard agarose gels separate DNA fragments from 25–0.1 kb, in which the size of the DNA fragments to be analyzed determines the percentage agarose to be used. For IgH and TCR-β gene rearrangements, 0.8% gels are routinely used. Molecules of linear double-stranded DNA migrate through the gel matrix at a rate that is inversely proportional to the $1\log_{10}$ of their molecular weight. The molecular weight of a fragment of interest is determined by comparing its mobility to the mobility of a DNA molecular weight standard; lambda DNA digested with Hind*III* is the marker of choice. We use the following procedure (*see* **Notes 2–6**):

1. Pour 0.8% agarose gel (20 cm × 20cm × 0.6cm) in 1X TBE (**Subheading 2.3., item 11**). (*See* **Note 2**).
2. Add ethidium bromide to the running buffer to a final concentration of 8 µg/L.
3. Load restriction enzyme digested DNA samples and marker.
4. Run the gel overnight at 20 mA at room temperature (*see* **Notes 3–6**).
5. Transfer the gel to UV transparent support film.
6. Photograph the gel under 300 nm UV light (*see* **Note 7**).

3.3.3. Transfer of DNA to a Solid Support Membrane

Southern blotting is the transfer of DNA fragments from a gel to a solid support membrane. There are three different transfer methodologies, electro-, vacuum-, or capillary transfer. In our experience, capillary transfer is a fail-safe mechanism that does not require additional equipment.

3.3.3.1. Capillary DNA Transfer

This is achieved under a high-salt buffer to promote the binding of the DNA to the membrane; immobilization of the DNA to the membrane is achieved through UV irradiation. After immobilization, the DNA is hybridized with a labeled probe, complementary to the gene segment of interest. Prior to transfer, the DNA must undergo three pretreatments—depurination, denaturation, and neutralization. Pretreatments are performed at room temperature, under gentle agitation, as follows:

1. Incubate gel in 0.2 *N* HCl for 20 min. The dye front will change to yellow/green. This step results in the depurination of the DNA, which will lead to strand cleavage and improves the transfer of high-molecular-weight DNA molecules after treatment with alkali (*see* **Note 8**).
2. Rinse briefly three times with ddH_2O to remove excess acid.
3. Incubate with denaturation solution (600 m*M* NaCl : 0.2 *N* NaOH) for 30 min. The dye front will turn blue. This steps denatures the DNA to give single-stranded DNA that has unparied bases and is suitable for subsequent hybridization analysis. It also breaks the DNA at depurinated bases to allow better transfer of large DNA fragments.
4. Repeat **step 3** to ensure complete denaturation of the DNA.
5. Incubate gel in neutralization buffer, pH 7.0 (3 *M* Tris-HCl, 0.5 *M* NaCl) for 30 min. This step brings the gel pH to <9.0 and allows the DNA to bind to the membrane. (This is critical if using nitrocellulose, but less critical if using nylon membranes.)
6. Rinse the gel in ddH_2O water. The DNA is ready to be transferred.

3.3.3.2. DNA Transfer by Capillary Action

Capillary transfer of DNA may be achieved in either an upward or downward direction. In an upward direction, a 500-g weight is placed on top of the

blotting stack to promote the capillary transfer. This weight crushes the gel and reduces the transfer of the DNA so that the procedure must be performed overnight. In a downward capillary transfer, weights are not used and the same efficiency of transfer may be accomplished in 4 h; a blotting system package from Schleicher and Schuell includes a transfer device that simplifies this procedure.

1. Cut the nylon membrane to the size of the gel. Prewet in ddH_2O.
2. Transfer to 20X SSC. Soak for a minimum of 15 min.
3. Stack blot paper, 20 sheets of GB004 under 4 sheets GB002 gel blot paper (Schleicher and Schuell).
4. In Pyrex dish with 500 mL 20X SSC, assemble the following in the order given: 1 sheet GB002 blot paper, nylon support membrane, DNA gel.
5. Excavate all air bubbles between the layers. Air bubbles within the blotting stack produces artifacts in that the DNA migrates around the entrapped air.
6. Transfer the gel assembly onto the blot paper stack from **step 2**. Make sure the gel assembly is perfectly aligned. It is difficult to remove air bubbles at this point. If air bubbles persist, return the gel assembly to the 20X SSC and repeat **step 4**. For blots containing low-molecular-weight DNA, it is important to manipulate the membrane minimally, as detectable transfer can occur almost immediately, and moving the membrane may cause "mobile" bands as streaks on the autoradiograph. The transfer will be complete after 4 h. Make sure the reservoir of 20X SSC does not dry out during the transfer.
7. Dissemble the blotting apparatus. Rinse the DNA membrane in 2X SSC. Do not discard the gel.
8. To immobilize the DNA, expose the DNA-membrane 120,000 Jcm^2 under 254 nm UV light. UV crosslinking is recommended for nylon membranes, as this leads to covalent attachment and enables the membrane to be stripped and reprobed several times.
9. Briefly examine the DNA-membrane under 254 nm UV. The presentation of the DNA on the membrane should mirror the ethidium bromide staining seen after electrophoresis. If radiolabeled probes are to be used, indicate DNA lanes and DNA marker positions with a number 2 pencil.
10. Prepare the DNA membrane for hybridization. Alternatively, membranes can be stored wrapped in Saran Wrap at 4°C for long-term storage (in our experience, Zetabind [CUNO] is a high-quality nylon membrane that generates minimal background noise after hybridization; also *see* **Note 8**).
11. Restain the gel overnight in 500 mL 1X TBE containing 5 μg ethidium bromide. Photograph the gel to confirm the transfer of DNA (*see* **Note 8**).

3.3.4. Hybridization Analysis

During hybridization, a single-stranded DNA molecule of defined sequence (the probe) base-pairs to a second DNA molecule that contains its complementary sequence (the target). The stability of the hybrid depends on the homology

between the probe and the target. Hybridization is sensitive and permits the detection of single-copy genes in complex genomes. The labeled probe is applied in a solution that promotes hybridization. After a suitable incubation period, the membrane is washed to remove the nonhybridized probe, leaving only the probe that is base-paired to its target. The hybridization process can be divided into three stages. First, the membrane is incubated in a prehybridization solution containing reagents that block nonspecific DNA binding sites to reduce background hybridization. Second, the labeled probe is added to the buffer and incubated overnight. During this stage, the probe not only binds to the target sequence, but to other related sequences. Third, the membrane is washed in a series of solutions that gradually remove the non-specifically bound probe. There are a number of reporter molecules that may be used to label the probes. In our experience, the use of radiolabeled probes remains the standard. Although there is the assumed inconvenience of working with radioactivity, the results are unquestionably superior to the various indirect detection methods. The detection limit for a radioactive probe with a specific activity of 10^8–10^9 dpm/µg is about 0.5 pg DNA (*see* **Notes 9** and **10**).

3.3.5. Random Primer Labeling of DNA Probes

A random primer reaction consists of probe, random DNA hexamers, and reaction buffer containing unlabeled dATP, TTP, dGTP, and ^{32}P-dCTP, during which a single copy per template is generated. The random hexamers anneal to the complementary targets on the probe and, on addition of a DNA polymerase, complementary nucleotides from the reaction buffer are incorporated to complete the new strand. We use the following steps:

3.3.5.1. Random Priming

1. Adjust 200ng DNA probe to volume of 8 µL with ddH_2O. Heat at 95°C for 5 min. This denatures the double-stranded probe to single-stranded DNA.
2. Place the denatured probe immediately on ice. This inhibits the DNA strands from reannealing.
3. To the ice-cold probe add:

Hexamers	4 µL
BSA	2 µL
RPRB	24 µL
^{32}P-adCTP(approx 3000 Ci/mmole)	20 µL
Klenow	2 µL (8 U)

4. Heat inactivate the enzyme at 65°C for 10 min. Incubate at room temperature overnight or at 37°C or for a minimum of 1 h.

3.3.5.2. Probe Purification

After the random primer reaction is complete, unincorporated nucleotides must be removed. This is a critical step—to ensure that a clean probe is hybridized with the DNA blots. There are many commercially available columns to accomplish this step. Our laboratory uses the Stratagene Push columns, which eliminates the need to use a centrifuge. The reader is referred to the manufacturer's instruction for this procedure.

3.3.5.3. Purified Probe

1. Determine the specific activity of the radiolabeled probe. Calculate the volume required to give 2×10^8 cpm. Aliquot volume with equal volume of deionized formamide.
2. Heat probe the formamide mix at 95°C for 5 min prior to adding to the hybridization buffer. Formamide destabilizes DNA duplexes, which, in addition to the heat, ensures that the probe is single stranded.

3.3.6. Hybridization

Most hybridization procedures are performed in hybridization ovens, which have surpassed the original sealed-bag-and-water-bath incubation. Hybridization is performed at 65°C.

3.3.6.1. Procedure

1. Preheat oven and hybridization buffer to 65°C.
2. Preincubate DNA membrane in 15 mL hybridization buffer for a minimum of 20 min.
3. Add the radiolabeled probe (*see* **Subheadings 3.3.5.1. and 3.3.5.2**). Do not allow the probe to track down the membrane, as this may cause the nonspecific probe to adhere to the membrane.
4. Hybridize overnight.
5. Preheat wash buffer A and wash buffer B.
6. Decant radiolabeled buffer from **step 3**.
7. Posthybridization washes are each for a minimum of 20 min: two with buffer A (low stringency), four with buffer B (high stringency). The high-stringency buffers should destabilize all mismatched heteroduplexes, so that hybridization signals are obtained only for the required sequences.
8. Rinse DNA membranes with ddH_2O to remove residual wash buffer.
9. Air-dry. Place on blot paper (GB004) and wrap in plastic. The DNA membrane is ready for autoradiography (*see* **Notes 9** and **10**).

Table 2
Molecular Weights of Routine Cross Hybridizing Bands Following Hybridization of J_H and $C_T\beta$ DNA Probes

*Sau*3a J_H rearrangements Southern analysis			
Enzyme	*Hind*III	*Eco*RI	*Bam*HI
x-hybridizing bands	3.5kb	26kb	11.5KB
*Bgl*II/*Pst*I CTβ rearrangements Southern analysis			
Enzyme	*Hind*III	*Eco*RI	*Bam*HI
x-hybridizing bands	—	—	17.5kb

3.3.6.2. Interpretation

A clonal gene rearrangement is defined by the detection of a single change in the germline pattern in at least two enzyme lanes or a double change within a single enzyme lane. A change is constituted either by the gain of a unique band or the loss of a germline band. The germline bands for $C_T\beta$ and J_H probes using the restrictive enzymes *Hind*III, *Eco*RI, and *Bam*HI are shown in **Fig. 1**. The *Sau*3a 2.5-kb J_H, and *Bgl*I/*Pst*I 400-bp $C_T\beta$ probe crosshybridize with predicted sequences to generate routine cross-hybridizing bands (*see* **Table 2** and **Fig. 2**). In addition, partial digest bands may be detected in the *Hind*III and *Eco*RI digests following hybridization with the $C_T\beta$ probe (*see* **Fig. 1,** also *see* **Notes 11** and **12**).

3.4. PCR-Based Techniques

The advantages of a PCR-based diagnostic test compared with a Southern analysis are that (1) quantitatively, much less DNA is required; (2) the integrity of the DNA is less critical; (3) the procedure requires less technical manipulations, whereby the turnaround times are reduced; and (4) a PCR-based assay exhibits a much greater level of sensitivity. There are two disadvantages. First, PCR assays are less robust than Southern analyses, in that PCR targets very specific regions of the genome. However, multiple primer pairs can be established to circumvent this problem as in the case of the t(14;18) test, in which specific primers are directed against regions in the MBR and mcr, which results in a high-quality test. Second, there is an overwhelming potential for contamination and the possibility of generating false positives. However, with rigid laboratory protocols, and separate processing and amplification laboratories, contamination can be kept to a minimum. Some laboratories control for

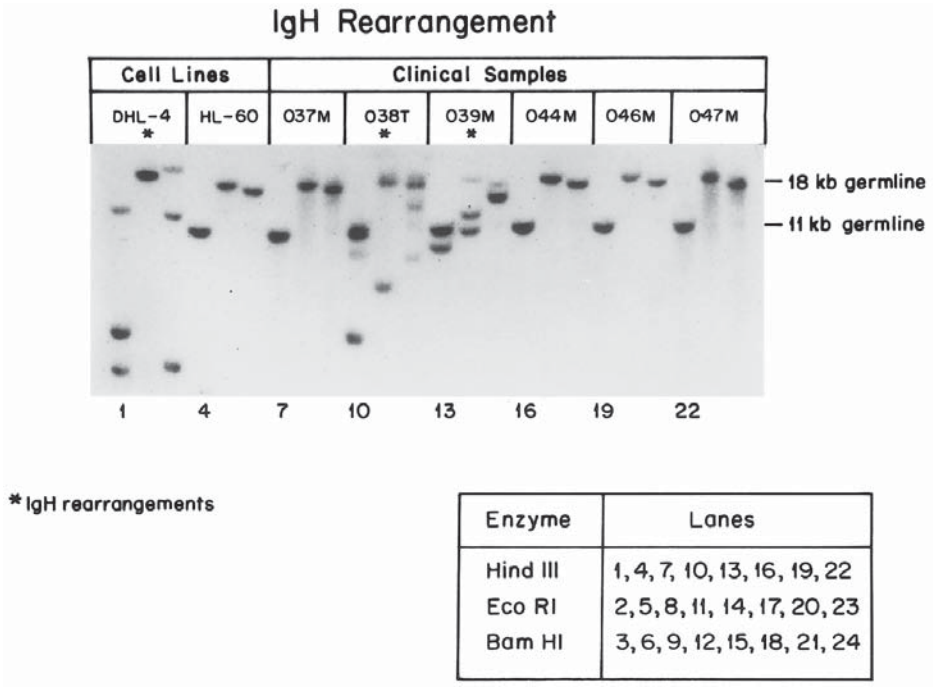


Fig. 2. Southern blot analysis of the IgH gene. Control DNA and DNA from six clinical samples were restricted and hybridized with the *Sau*3a J_H DNA probe. Clonal IgH gene rearrangements were detected in DNA isolated from DHL-4, positive control cell line, and clinical samples 038T and 039M; germline bands were detected in DNA isolated from HL-60, negative control cell line, and the remaining clinical samples.

contamination by using dUTP during PCR; all successive PCR reaction mixtures are treated with uracil-DNA glycosylase to degrade uracil-based amplification products, thereby eliminating potential contaminating PCR products. Our laboratory does not use dUTP, but instead uses stringent laboratory practices to include exclusive use of Art plugged tips (Molecular Bio-Products, San Diego, CA), frequent change of gloves and benchtop diapers during preparative procedures, single-use aliquots, and separate pre- and amplification laboratories. But perhaps the single most important factor is the ability of the technician to diligently adhere to rigid laboratory practices. The contamination rate in our laboratory is <1 : 2500.

Detecting a mutation that occurs within a nontranscribed region of a gene is achieved with a standard PCR assay. A standard PCR-based test consists of amplification and detection. During amplification, the DNA template is incubated at three different temperatures. First, the denaturation step is achieved at

94°/95°C to generate single strands of DNA. Second, the temperature is lowered to the annealing temperature during which the primers hybridize to their complementary targets. This is the most critical temperature of the reaction and is primer-specific, determined by the theoretical melting temperature (*Tm*) at which the primer will dissociate from its complementary template. An approximate working *Tm* can be calculated as follows: $Tm = [2°C \times (A + T) + 4°C \times (G + C)]$, in which more energy is required to dissociate cytosine and guanine bases due to the triple hydrogen bonding, compared with the double hydrogen bonding of adenine and thymidine. An annealing temperature usually approximates to be 5°C lower than the *Tm* of the primer. Third, the extension phase of the reaction is at 72°C, the optimal temperature for *Taq* polymerase. During this phase, a complementary oligonucleotide chain is synthesized. On completion of the reaction, the amplified products are detected as discussed in the following section.

3.4.1. DNA-Based PCR

For each test, the appropriate positive and negative controls must be used. Our laboratory routinely uses the positive control DNA at sensitivity dilutions of $1:10^4$ and $1:10^5$; it is important to establish these dilutions to reduce the potential source of contaminating positive PCR products. A water-blank control is also incorporated into the assay, which acts as a critical control indicator to monitor potential DNA carryover. This reaction tube is set up as the final tube during an assay and contains all reagents in the absence of DNA. Therefore, detection of amplified products from this sample indicates carryover, and the entire test must be repeated. At least two primer pairs must be used for each assay. First, the primer set targeting the mutation or gene rearrangement of interest, and second an internal control primer-set targeting a nonmutated/nonrearranged DNA sequence. Some assays include a second nested round of PCR, which increases both the specificity and sensitivity of the assay. We use the following procedure:

1. Allow aliquots to thaw on ice: l0 m*M* dNTPs, l0X PCR buffer + $MgCl_2$, l0 pmol 5′ primer, l0 pmol 3′ primers.
2. Calculate the volume of patient DNA to give l µg DNA.
3. For 50 µL reaction volume, calculate the volume of ddH_2O to bring the entire reaction volume to 42 µL (DNA + ddH_2O = 42 µL).
   The following steps are performed on ice:
4. Prepare master reaction mix for each primer set to be used as follows (to minimize pipetting errors, master reaction mixes are prepared, and then aliquoted into each reaction tube): 5 µL l0X PCR buffer plus MgC_{12} (concentration is dependent upon primer sequence) l µL of l0 m*M* dNTPs, l µL l0 pmol 5′ primer, and l µL l0pmol 3′ primer.

5. Place labeled thermal cycler tubes on ice.
6. Add calculated ddH_20 volumes to each tube.
7. Aliquot 8 µL of master reaction mix into tubes.
8. Add calculated DNA volumes to each appropriate tube.
9. Take tubes to PCR lab.
   The following steps are performed in the PCR lab:
10. Place tubes in the thermalcycler.
11. Perform a hot start procedure: 95°C 2 min ramp to 4°C. A hot start is recommended to denature the template and to dissociate nonspecific primer annealing that may have occurred.
12. Remove tubes from thermacycler. Place on ice for 5 min.
13. Add 4 U *Taq* polymerase to each tube.
14. Place tubes into the thermacycler and run amplification program, e.g., 94°C 15 s annealing temperature 15 s at 72°C × 30 s for a total of 25 cycles at 72°C × 10 min. Ramp to 4°C.
15. Remove tubes and place at 4°C.
    The following are the second nested rounds of PCR:
16. Allow aliquots to thaw on ice: 10 m*M* dNTPs, 10X PCR buffer plus $MgCl_2$, l0 pmol 5′ primer, l0 pmol 3′ primers.
17. Label reaction tubes for second round of PCR.
18. Prepare a master reaction mixes for each tube: 41 µL ddH_20
19. 5 µL l0X PCR buffer + $MgCl_2$, 1, µL l0mM dNTP's, lµL l0pmole 5′ primer, l µL 10pmol 3′ primer
20. Place labeled thermacycler tubes on ice.
21. Aliquot 49 µL of master mix into second round tubes.
    The following steps are performed in the PCR lab:
22. Pull first-round reactions from the refrigerator. Carefully, add l µL of first PCR product to the *correct* second-round tube. Second-round tubes must be on ice.
23. Place tubes in the thermacycler.
24. Perform hot start.
25. Remove tubes from thermacycler and hold on ice for 5 min.
26. Add 4 U *Taq* polymerase to each tube.
27. Return tubes to the thermacycler run cycle program.
28. Remove tubes and hold 4°C until detection analysis.

3.4.2. RT-PCR-Based Assays

Detecting mutations that occur within transcribed regions of genes is achieved by using an RT-PCR based assay, involving a preamplification step in which mRNA is reverse transcribed into cDNA prior to cycling, described as follows.

1. Allow aliquots to thaw on ice: l0 m*M* dNTPs, l0X PCR buffer and l0 pmol 3′ primer.
2. Calculate the volume of patient RNA to give l µg RNA and add to a clean tube.

3. For a 50-µL reaction volume, calculate the volume of ddH_2O necessary to bring DNA+ddH_2O = 13 µL
4. Prepare master reaction mix for each 3′ primer as follows: 2 µL 10X PCR buffer, 2 µL 10 m*M* dNTPs, 1 µL 10 pmol 3′ primer, and 1 µL Rnase inhibitor (40 U/µL).
5. Place labeled thermacycler tubes on ice.
6. Add calculated ddH_2O volumes to each tube.
7. Aliquot 6 µL of master reaction mix into tubes.
8. Add calculated RNA volumes to each appropriate tube.
9. Add 200 U reverse transcriptase to each tube.
10. Incubate at 42°C for 1 h.
11. Heat inactivate enzyme at 95°C for 5 min.
12. Transfer to ice.
13. Prepare master reaction mixes for each primer-set: 70 µL ddH_2O, 8 µL l0X PCR buffer plus $MgCl_2$, and l µL l0 pmole 5 = primer.
14. Aliquot 79 µL of reaction mix into reverse-transcribed reactions.
15. Perform hot start.
16. Remove tubes from thermacycler and hold on ice for 5 min.
17. Add 4 U *Taq* polymerase per tube.
18. Return tubes to the thermacycler run cycle program
19. Remove tubes and hold 4°C until detection analysis is performed.

3.4.3. Detecting PCR Products

Detecting amplified products can be achieved by using several different methods, which is determined by the level of sensitivity required of the PCR assay performed. A DNA-based nested PCR assay is sensitive to $1:10^5$ following routine agarose gel electrophoresis with ethidium bromide staining (*see* **Fig. 3**). The same order of sensitivity from an RT-PCR assay can be achieved from a single round of PCR followed by a solution hybridization of a radiolabeled nested probe (*see* **Fig. 4**): A single round of DNA-based PCR with solution hybridization of a radiolabeled probe is sensitive to approximately $1:10^3$. A nested RT-PCR-based assay increases the sensitivity to $>>1:10^6$, but this can become problematic in terms of controlling contamination; care must be taken to reduce the number of first-round amplification cycles to adjust the sensitivity of the assay within the realms of controllable contamination.

By using solution hybridizations, you can detect the labeled probe by determining the nature of the reporter molecule. As mentioned, radiolabeled probes are superior in terms of sensitivity and generate low background levels. Regardless of the reporter molecule used, detection can be achieved following either dot blotting or gel electrophoresis. Dot blotting does not allow for molecular size determination of the PCR products and involves the transfer of PCR products to a solid support membrane, hybridization with the labeled

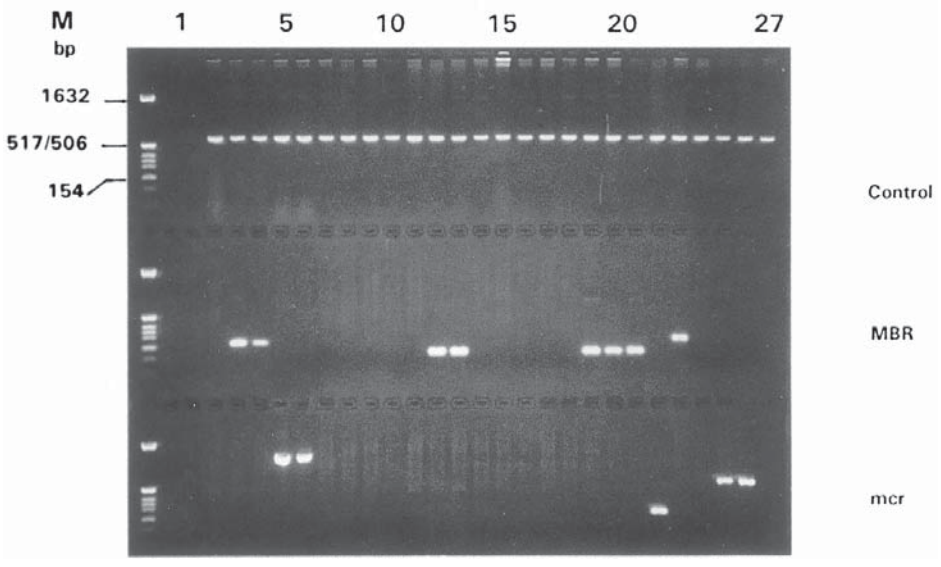


Fig. 3. DNA-based nested-PCR t(14;18) diagnostic assay. Detection of amplification is achieved through ethidium bromide–DNA intercalation visualized under 300 nm UV light following electrophoresis in 2% agarose gel. M = pBR322 *Hinf* I digest (1632, 517–506, 396, 344, 298, 154, and 75 base pairs). MBR = major breakpoint region; mcr = minor cluster region.

nested probe, and detection of the annealed probe. If the molecular weight of the PCR product is required, electrophoresis must precede detection. Electrophoresis may be achieved through high-percentage (2% agarose gels or 6% denaturing acrylamide gels (for PCR products < 1kb). For radiolabeled probes, the results would be detected following autoradiography, where the gels are exposed directly to the X-ray film at –70°C. High-quality results are achieved when acrylamide gels are vacuum-dried under heat prior to exposure; agarose gels, by comparison, are not dried prior to exposure and the resulting autographs are of lower quality.

The detection method of choice in the diagnostic laboratory is the visualization of DNA intercalated with ethidium bromide under 300 nm UV light following agarose gel electrophoresis. The reader is referred to Sambrook et al. ***(31)*** for solution hybridizations and acrylamide gel electrophoresis procedures, as these are not considered to be standard diagnostic protocols.

3.4.4. Agarose Gel Electrophoresis of PCR Products

The percentage agarose gel is determined by the size of the PCR products to be analyzed. Routinely, a 2% wt/vol agarose gel is used to efficiently separate

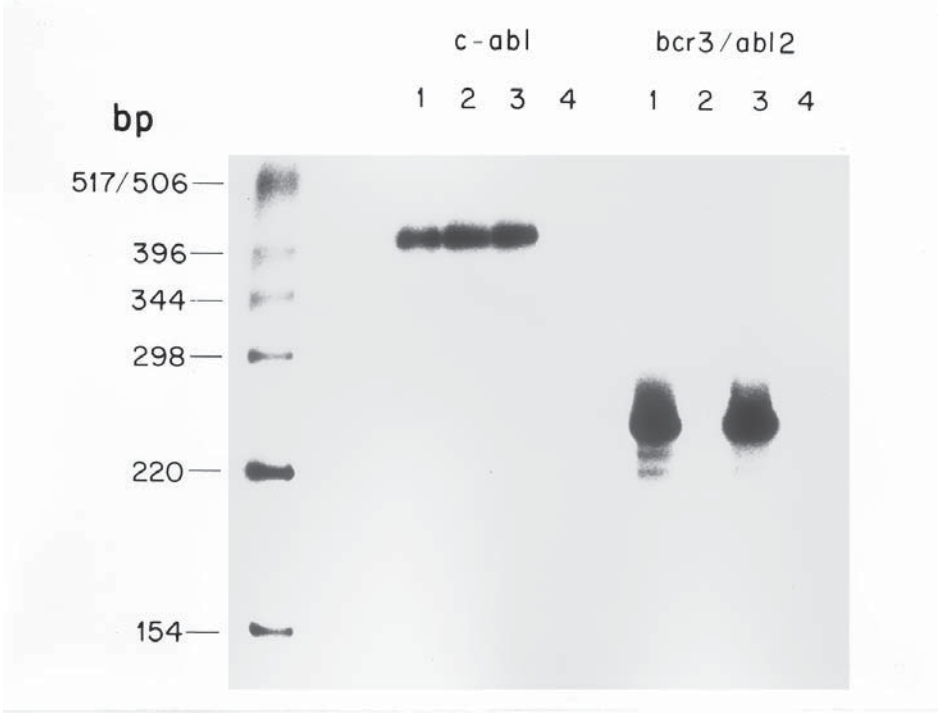


Fig. 4. RT-PCR *bcr/abl* analysis of nodal cells. Solution hybridization of 5′ end-labeled nested oligonucleotide probes with PCR products analyzed on 8% polyacrylamide gel. *c-abl*:internal *c-abl* probe for quality control panel for isolated RNA. *bcr3/abl2* : junction specific probe to detect *bcr* exon 3/*abl* exon 2 mRNA fusion sequences. **Lane 1:** K562 Ph chromosome-positive cell line; **lane 2:** HL-60 Ph chromosome-negative control cell line; **lane 3:** patient sample; **lane 4:** blank control. 3′ End-labeled pBR322 *Hinf*1 digest was used for the base pair (bp) size marker.

linear DNA within the range of 0.1–2 kb. Ethidium bromide is added directly to the agarose gel at a concentration of 20 µg /350 mL gel-vol, for short-term (4–6 h) electrophoresis.

3.4.4.1. Preparing Amplified Products for Gel Electrophoresis

1. Take amplified product tubes and to each add gel-loading buffer for final lX concentration. Pipet to mix.
2. Load one-fifth of PCR products on gel. Load l µg molecular-weight marker e.g., pBR322 *Hinf* I digest (75bp–1632).
3. Run gel at 30–40 mA for approximately 4.5 h, which allows for sufficient separation of the size marker.
4. Store all remaining PCR products at –20°C until results have been interpreted. The PCR products are discarded at this time.

3.4.4.2. Photodocumentation

1. Expose the gel to 300 nm UV light and capture the image on Type 57 film (Polaroid, Cambridge, MA).
2. Remove gel and discard into solid ethidium bromide waste container.

3.4.5. Analyzing the PCR Assay

The ethidium bromide will intercalate double stranded DNA and will fluoresce under 300 nm UV light. A fluorescent band shows the presence of an amplified product; typical results generated from our t(14;18) assay is shown in **Fig. 3**.

1. Check that all the controls amplified with the correct primers, as in the following table:

DNA	Control primers	Test primers
Water blank	–	–
Negative-control DNA	+	–
Positive DNA	+	+

2. If any of the controls show a banding pattern that differs from the table, none of the patient sample results can be interpreted (report as technical problems). Repeat the assay if sufficient DNA is available.
3. In the patient samples, a PCR product must be present with the control primers to show that amplifiable DNA was present in the reaction tubes. If there is a control PCR product present, results are reported as negative in the absence of a band generated with the test primers. If a PCR product is generated with the test primers, the results are reported as positive.
4. If there is no amplification for a patient sample with either the control or test primers on the gel, the results are reported as technical problems. Repeat the assay for this patient if sufficient DNA is available (*see* **Notes 13** and **14**).

4. Notes

1. Quality of nucleic acids
   a. Optical density measurements: As we stated, the A_{260}/A_{280} ratio is used as the standard indicator of nucleic acid purity. However, our laboratory routinely scans nucleic acid isolates over A_{320}–A_{200} nm wavelengths to ascertain the overall purity of the sample. As shown in **Fig. 5**, DNA samples have A_{260}/A_{280} values of 1.75 and 1.74, respectively, in the absence of phenolic (A_{270}) contaminants and particulate matter (A_{320}). However, in **(B)**, the A_{260}/A_{230} ratio approaches 1.0. DNA samples with high A_{230} values will not be digested with the restriction enzyme *Hind*III (*Eco*RI and *Bam*HI are unaffected), nor will the material be amplifiable. Thus, although our laboratory determines the

Lane	Sample	Lane	Sample	Lane	Sample
1	H_2O blank	10	Patient C BM	19	Patient J PB
2	K562 –ve control	11	Patient D BM	20	Patient J BM
3	RL MBR + ve control $1:10^4$	12	Patient E BM	21	Patient J Core
4	RL MBR + ve control $1:10^5$	13	Patient E Core	22	Patient K BM
5	DHL-26 mcr + ve control $1:10^4$	14	Patient F BM	23	Patient L BM
6	DHL-16 mcr + ve control $1:10^5$	15	Patient G BM	24	Patient M PB
7	Patient A BM	16	Patient G Core	25	Patient M BM
8	Patient A Core	17	Patient H BM	26	Patient M Core
9	Patient B BM	18	Patient I Core	27	Patient N BM

BM, bone marrow; PB, peripheral blood.

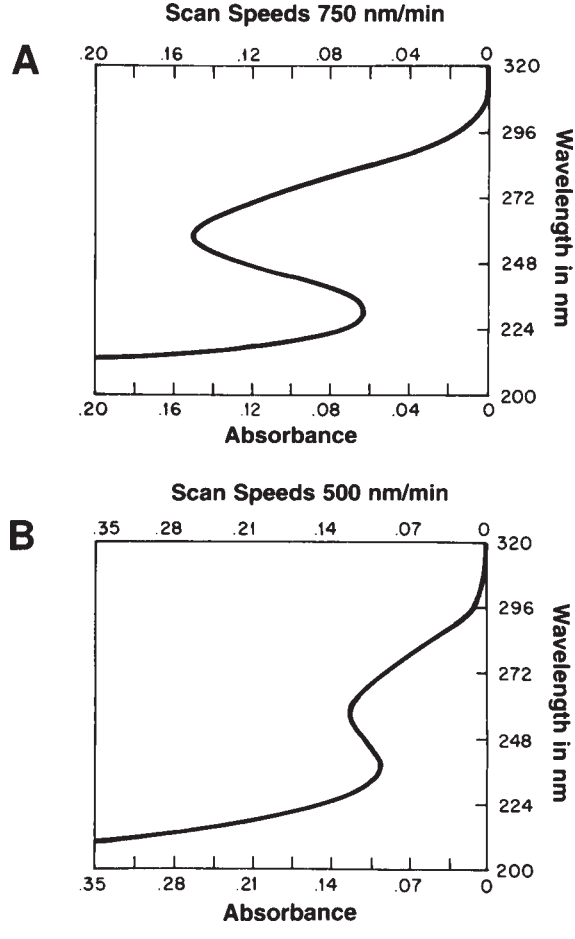
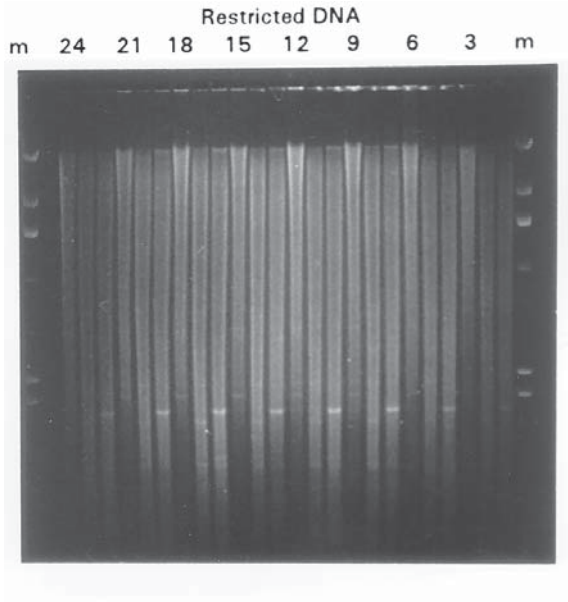


Fig. 5. Spectroscopic absorption A_{320nm}–A_{200nm} scans of isolated nucleic acids to assess sample quality. Scans in **(A)** and **(B)** are compliant with the A_{260nm}/A_{280nm}, A_{260nm}/A_{270nm}, and A_{320nm}/A_{260nm} indicator ratios, whereas only the scan in **(A)** is compliant with the A_{260nm}/A_{230nm} indicator ratio.

classic purity A_{260}/A_{280} ratio, we view the A_{260}/A_{230} ratio to be a more critical indicator of nucleic acid purity.

b. Southern blot analysis: For Southern analyses, it is advisable to run a preparative gel prior to restriction endonuclease digestion to confirm the integrity of the isolated DNA. Aliquot 1 µg DNA and adjust volume with ddH_2O to 10 µL. Incubate at 37°C with 1 U RNase A. Add loading dye to 1X concentration. Add ethidium bromide to running buffer to a final concentration of 8 µg/L. Electrophoresis on 0.8% gel at 20 m/A for 4 h against lambda DNA molecular-weight marker. Visualize under 300 mn UV light to determine the integrity of DNA. NB: Undegraded DNA will produce a single band of high molecular weight (which migrates a very short distance at the top of the gel), whereas degraded DNA will produce a smear. Degraded DNA is not suitable for Southern analysis.

2. When gels are poured, ensure that any air bubbles are removed. Air trapped within the gel will generate both electrophoretic and blotting artifacts.
3. Gels run for extended periods, e.g., overnight, should use Tris/borate/EDTA (TBE), as the Tris/borate has a significantly greater buffering capacity compared with Tris/acetate (TAE), which is more easily exhausted during prolonged runs.
4. Ethidium bromide is an intercalating dye. It allows the visualization of DNA when exposed, optimally, to 300 nm UV light. It intercalates between stacked bases of nucleic acids and fluoresces red-orange when illuminated under 260–360 nm UV light. It is positively charged and will migrate toward the cathode; for this reason, the dye is added to the running buffer during prolonged electrophoresis. Furthermore, if high background levels of ethidium bromide are transferred during blotting, it will (1) be difficult to visualize the restricted DNA under UV light and (2) it will interfere with the kinetics of the hybridization reaction.
5. The resistance in the gel is inversely proportional to the crosssectional area and the ionic strength of the buffer, of which the gel provides most of the resistance in the circuit and the voltage applied to the gel. For a given current, decreasing either the thickness of the gel and the overlying buffer, under constant ionic strength, will increase the resistance and consequently increase the voltage gradient across the gel and the electrophoretic mobility of the sample. A practical upper limit to the voltage is usually set by the ability of the gel apparatus to dissipate heat. The depth of the running buffer over the gel should be 3–5 mm. Excessive buffer will decrease DNA mobility and cause excessive heat within the gel, promoting band distortion.
6. Electrophoresis is complete when the loading dye front has migrated 75% the length of the gel.
7. Critical indicator I—completeness of digestion: after electrophoresis, the gel is photographed to determine the completeness of DNA digestion. The degree of digestion will depend on the purity of the DNA. Contaminants found in some preparations of DNA (e.g., protein, phenol, chloroform, ethanol, SDS, EDTA, high salt concentration) may inhibit the reaction. The decreased reaction effi-



Enzyme	Lanes
HindIII	1,4,7,10,13,16,19,22
EcoRI	2,5,8,11,14,17,20,23
BamHI	3,6,9,12,15,18,21,24

Fig. 6. Restricted DNA intercalated with ethidium bromide and photographed under 300 nm UV light following electrophoresis through 0.8% agarose gel. Each endonuclease will generate characteristic heavy-stained fragments (repetitive sequences), detection of which is used as a critical indicator to confirm complete digestion.

ciency may be overcome by adding more enzyme and increasing the length of the incubation period. When digestion is complete and the DNA is stained with ethidium bromide and visualized under 300 nm UV light, the DNA appears as a smear the length of the gel, with each enzyme displaying characteristic intensely stained bands of DNA within the smear (*see* **Fig. 6**). If the DNA does not generate this pattern, then first the DNA may have been underloaded. In this case, the

entire smear will be of a lower intensity. If the DNA is partially digested or undigested, a disportionate amount of stained DNA will be in the upper portion of the gel and these characteristic stained bands will be lost. If the DNA was degraded, there will be an absence of high-molecular-weight DNA in the upper portion of the gel and a greater amount of low-molecular-weight DNA. This is a critical evaluation, because the extent of DNA restriction will determine the molecular weight of the DNA fragment containing the gene segment of interest.

8. Critical indicator II—efficiency of transfer: A rate-limiting step is the transfer of DNA to the solid support membranes, as the sensitivity of detection is determined by the amount of DNA present on the membrane. If the gel and the blotting stack are of the same dimensions, the buffer will be forced to migrate through the agarose gel and transfer the DNA. Poor transfer is seen when the gel is smaller than the blot stack, in which case, the buffer preferentially travels through the blot paper. Furthermore, insufficient depurination will reduce the transfer of high-molecular-weight DNA. The length of time the gel is incubated in the 0.2-*N* HCl is critical; excessive depurination will fragment the DNA beyond sequence recognition that will alter the hybridization of complementary probes.
9. Autoradiography produces a permanent image on X-ray film of the distribution of radioactive probe from hybridization . ^{32}P is a beta particle emitter; to increase the efficiency with which these beta particles are detected, an intensifying screen is placed behind the X-ray film and the hybridized DNA membrane. A signal will be enhanced approximately fivefold when held at –70°C.
10. Critical indicator III—efficiency of hybridization: One of the most frequent problems that influence sensitivity is the specific activity of the probe. If the specific activity is too low, the 5% positive sensitivity spike will not be detected. High background levels can be generated due to dirty probes, insufficient prehybridizations, and interruption of constant 65°C temperature conditions.
11. J_H probes: The *Sau*3a 2.5-kb J_H-cloned DNA fragment has been the standard probe used to detect IgH chain gene rearrangements. This probe spans J2–J6 sequences and extends 230 bp into the 3′ flanking region. The limitations of this probe are apparent when (V)DJ recombinations involve the J6 gene segment. Using the *Sau*3a J_H probe to detect clonal (Vn) DnJ6 gene rearrangements generates a relatively unstable hybrid, as the majority of the length of the probe remains unhybridized; as the percentage contribution of the DNA derived from the malignant B-cell clone diminishes, the sensitivity of detection also is seen to decrease. A Southern blot analysis routinely has a 5–10% sensitivity. For clonal (Vn) DnJ6 gene rearrangements, Southern blot sensitivity with the *Sau*3a J_H probe has been demonstrated to be as low as 40% ***(32)***. The IgHJ6 DNA probe described by Beishuizen et al. ***(32)*** circumvents this problem, and is now considered the standard probe to detect IgH gene rearrangements. The probe is complementary to 1020 bp of the J6 3′ flanking region. Thus, regardless of the J region involved in the recombination of the heavy chain, the sensitivity of detection remains constant at 5–10%.

12. IgH hypervariable polymorphisms (HVP): Germline polymorphisms of the IgH chain are caused by the presence of variation in the number of short tandem repeats (VNTRs) located in the J_H 5′ flanking region. The repeated segments are 50 bp long and the polymorphic variation is stably inherited ***(33)***. Four common alleles have been identified, which differ by up to 8 repeats, and two rare alleles have been shown to differ by up to 24 repeats (1020 bp). *Eco*RI and *Hind*III endonuclease restriction sites are located 5′ to the HVP region (*see* **Fig. 1**). In the presence of a polymorphism, two different germline *Eco*RI and *Hind*III fragments are generated on restriction. However, this is not a concern for *Eco*RI restriction, as the germline J_H *Eco*RI fragment is 18 kb, and the largest polymorphic variant (19.2 kb) will remain undetected because DNA fragments of this size are not resolved following electrophoresis in 0.8% agarose gels. In the case of *Hind*III restriction, the germline J_H *Hind*III fragment is 11 kb and in the presence of the largest (and rarest) VNTRs, two germline fragments of equal intensity will be visualized. Accordingly, <0.5% of patients' samples analyzed in our laboratory have displayed a *Hind*III polymorphism, in which the germline band presents as a doublet. The HVP will not influence clonal (V)DJ detection, as the HVP is lost during D-J splicing.
13. Critical indicator parameters for PCR-based assays: Generally, amplification efficiency is dependent on each component in the reaction tube, the quality and quantity of the target nucleic acid, the concentration of the enzyme, and the deoxynucleotide triphosphates and primer-sequence dependent magnesium chloride concentrations, buffering conditions, and annealing temperatures. Initial startup of a PCR-based assay must include optimization of the reaction for each primer pair used in the assay in order to standardize the assay and determine the level of sensitivity. Assuming rigid adherence to optimized reaction conditions, amplification failures with control primers will indicate either the presence of contaminants in the reaction or insufficient target DNA. The latter is probably unlikely if the DNA was quantitated prior to amplification. Thus, amplification within the control regions is the first critical indicator. A second indicator tests for carryover contamination. This is accomplished through the water-blank reaction tubes, which do not contain target nucleic acids. The detection of PCR products in these reactions for any of the prime pairs voids the entire assay. The assay must be repeated using fresh aliquots of reagents.
14. t(14;18) in "normal" cells: The *bcl*-2 gene encodes a protein involved in apoptosis, and dysregulation of the gene confers prolonged survival on the cell. The t(14;18) translocation occurs as an early mutational event in pre-B cells within the multistep cascade of genetic changes observed during carcinogenesis. The translocation alone does not confer a malignant phenotype on the cell but instead allows the accumulation of genetic changes necessary for the malignant transformation to occur. Because the PCR assay is very sensitive, false-positive results are a problem. Cells bearing the t(14;18) translocation have been found in hyperplastic tissue in the absence of lymphoma ***(34)***; they have also been found

at a frequency of (1 : 105 in normal circulating B cells in six of nine healthy individuals ***(35)***. To detect these rare cells, however, the peripheral blood must be fractionated and the assay must be performed on DNA extracted from a purified B-cell population. Such purification procedures are not performed in diagnostic laboratories, but instead DNA is extracted from a total nucleated cell population after red blood cell lysis. Under these conditions, false-positives related to the presence of nonmalignant, t(14;18)-bearing cells is rare.

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6

Detecting Monoclonal Immunoglobulin and T-Cell Receptor Gene Rearrangements in B- and T-Cell Malignancies by Polymerase Chain Reaction

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1. Introduction

B cells undergo gene rearrangement of one of their immunoglobulin heavy-chain genes at an early stage in B-cell development. During rearrangement of the immunoglobulin heavy-chain gene (*IgH*), a variable gene segment (*V*) is joined to a diversity gene segment (*D*), and then subsequently this complex is recombined to a joining gene segment (*J*). Nucleotides are added and removed at random at the *V–D* and *D–J* junctions ***(1)***. Similarly, early in development of T cells, the T-cell receptor (*TCR*) genes rearrange. In the case of the T-cell receptor γ (*TCR*γ) gene, the *V*-gene segment is brought into juxtaposition of a *J*-gene segment. Nucleotides are then added and deleted at random at the *V–J* junction ***(2)***. It is these gene rearrangements that are responsible for the immune repertoire. Usually only one of the chromosomes will rearrange and the other remains in the germ-line configuration. Although, if the first rearrangement is ineffective then the other chromosome may rearrange. The *IgH* and *TCR*γ rearrangements will vary in DNA sequence and usually in size between lymphocyte clones and a normal individual will have a very large number of different *IgH* or *TCR*γ rearrangements.

Malignant populations of B and T cells have been detected conventionally using morphology. Sometimes it is difficult to obtain a firm diagnosis from samples of suboptimal morphology, from very small samples, or from samples with complexity of cell populations. Most malignant lymphoproliferative disorders are monoclonal proliferations of cells. Monoclonal populations of B or T cells will have a monoclonal gene rearrangement at the *IgH* and/or the *TCR*γ

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gene, which can be used as a marker for monoclonality. Monoclonal populations have been detected at the DNA level in the past using Southern blotting, which has a limit of detection of 1–5% ***(3–7)***. However, this method requires a large amount of DNA of high integrity and is time consuming.

Polymerase chain reaction (PCR) ***(8)*** methods have since been developed to detect monoclonal gene rearrangements in a wide range of samples. These methods are more sensitive and faster than Southern blotting; they only require a small amount of DNA for analysis and can be used on partially degraded and archival samples. Although Southern blotting is still needed for detection of some rearrangements, the method is inappropriate for a routine diagnostic laboratory. The *IgH* gene contains conserved DNA sequences in most *V*-gene segments and most *J*-gene segments. These segments provide a DNA sequence for which consensus PCR primers can be designed. Several groups have designed consensus primers for the *IgH* gene ***(9–12)***. In the case of the *TCR*γ gene it is more difficult to design consensus primers, and therefore a mixture of PCR primers are used which cover most of the *V* and *J* genes ***(13–15)***. The PCR primers span the most variable region of the *IgH* and *TCR*γ genes.

1.1. Clinical Applications

1.1.1. Diagnosis

The methods described as follows can be used to help diagnose cell populations for B- or T-cell malignancy and can be performed on DNA from a wide range of different tissue types, e.g., bone marrow aspirates and trephines, peripheral blood, paraffin sections of all tissues, lymph node biopsies and aspirates, skin biopsies, pleural effusions, and ascitic fluid.

In practice, most leukemias and lymphomas can be diagnosed using the faster and more appropriate methods of histology and cytology. However, there are cases where it is difficult to make a confident diagnosis due to uncertain morphology, uncertain lineage or low tumor burden in the sample. This is particularly the case for follicular lymphoma, aspirates, and skin and gastric biopsies, where it may be uncertain whether the diagnosis is lymphoma or reactive. It is in these cases that the PCR tests described here, when taken in conjunction with morphology and immunophenotyping, can aid in diagnosis. For example, monoclonal T-cell PCR products can be detected in samples from patients with Sezary's syndrome and mycosis fungoides, which can otherwise be notoriously hard to diagnose by conventional methods. However, it is important to keep in mind that monoclonality has sometimes been detected in conditions in which distinction from malignancy is blurred and in cases where conditions are clearly not malignant (reviewed in ***16***).

1.1.2. Detection of Residual Disease

Although morphology can detect malignant cells at diagnosis in acute lymphoblastic leukemia, our laboratory has recently shown that monoclonality tests can be useful in detecting residual disease in marrow samples taken during induction therapy of patients with childhood acute lymphoblastic leukemia that are in morphological remission. The method has a limit of detection of 0.1% but is nonquantitative ***(17,18)***. Other methods for detection of residual disease can more quantitatively assess leukemia after treatment and, although very labor intensive, the methods allow accurate prediction of patient outcome (*see* Chapter 7, this volume). We have also reported four cases where monoclonality tests would have detected leukemia several months before morphological and clinical diagnosis in four children who first presented with remitting marrow failure ***(19)***.

1.2. Sensitivity and Specificity

The methods described as follows are theoretically capable of detecting a single rearranged immunoglobulin or *TCR*γ gene ***(20)***. However, in a clinical sample, the sensitivity of detection of monoclonal gene rearrangements is entirely dependent on the proportion of the normal polyclonal gene rearrangements present in the sample. In peripheral blood, the limit of detection for IgH rearrangements is approximately 5–10% ***(20)***, 1–5% in normal bone marrow (unpublished data), and 0.1% in bone marrow after 5 wk induction therapy for childhood acute lymphoblastic leukemia ***(18)***. In some samples, (e.g., a skin biopsy) where very few normal lymphocytes may be present, the limit of detection of a monoclonal population may be much lower than 0.1%, although accurate estimates would be difficult to obtain.

The sensitivity of these tests is also determined by the ability of the primers used to detect gene rearrangements. The IgH primers used here are in the framework 3A (FR3A) region and can detect approximately 90% of lymphoblastic leukemias and 75% of lymphomas. The tests only detect approximately 60% of myelomas, which may be due to the fact that somatic hypermutation has occurred in the malignant cells thus making it less likely that the PCR primers will bind to the conserved regions ***(21)***. Therefore, there will be some false negatives. Other groups have used different consensus primers in the FR3A region and have reported similar overall detection rates ***(22–24)***. A combination of different sets of primers to different framework regions can increase the detection rates of some tumor subtypes such as diffuse large-cell lymphoma ***(25)***.

Trainor et al. ***(13)*** detected 11/11 cases of T-lymphoproliferative disease using the TCRγ primers described in this chapter. In a larger study of 55 T-cell

lymphomas, Diss et al. ***(26)*** reported *TCR*γ gene rearrangements in 78% using the primers described by McCarthy et al. ***(15)***.

IgH and *TCR*γ gene rearrangements may result from monoclonal gammopathies (reviewed in ***16***) and careful clinical interpretation must be made. Specificities of 100% have been reported in studies of *IgH* ***(27)*** and *TCR*γ ***(28)*** gene rearrangements. A study of 54 node aspirates showed that the specificity of diagnosis using *IgH* gene rearrangement was 97% ***(20)***.

2. Materials

All chemicals, unless otherwise indicated, were obtained from Sigma Chemical Co., St. Louis, MO.

2.1. DNA Extraction

2.1.1. Peripheral Blood, Bone Marrow Aspirates and Fresh or Frozen Lymph Node Biopsies

1. Wizard DNA Purification Kit (Promega Biotech, Madison, WI, cat. no. A1120).
2. Isopropanol.
3. Ethanol 70%.

2.1.2. Lymph Node Aspirates and Pleural Effusions

1. Sterile saline 0.8% NaCl.
2. Lysis buffer: 0.32 *M* sucrose, 10 m*M* Tris-HCl (pH7.5), 5 m*M* $MgCl_2$, 1% Triton X-100.
3. Extraction buffer: 50 m*M* KCl, 10 m*M* Tris-HCl pH8.3, 0.1 mg/mL gelatin, 0.45% NP40, 0.45% Tween-20.
4. Proteinase K 1 mg/mL.

2.1.3. Paraffin Sections

1. Xylene.
2. Ethanol 100%.
3. Extraction buffer: 50 m*M* KCl, 10 m*M* Tris-HCl pH8.3, 0.1 mg/mL gelatin, 0.45% v/v NP40, 0.45% v/v Tween-20.
4. Proteinase K, 1 mg/mL.

2.1.4. Bone Marrow Trephines

1. K_2EDTA 1 mg/mL in saline.
2. Sterile saline 0.8% NaCl.

2.2. PCR

1. 5X PCR buffer: 335 m*M* Tris-HCl pH8.8, 83 m*M* $(NH_4)_2SO_4$, 2.25% Triton X-100, 1 mg/mL gelatin, 10 m*M* $MgCl_2$, 500 µ*M* each of dATP, dCTP, dGTP, dTTP.

2. AmpliTaq Gold™ 5U/µL (Perkin-Elmer, Applied Biosystems, Foster City, CA).
3. IgH Primer sequences ***(9)*** (*see* **Notes 1** and **2**).
   a. V region: FR3A (100 ng/µL) 5′ ACACG GC (C/T) (G/C) T GTATT ACTGT 3′
   b. J region: VLJH (100 ng/µL) 5′ GTGAC CAGGG T(A/G/C/T) CCT TGGCC CCAG 3′
4. *TCR*γ Primer sequences ***(13)***.

   There are two families of primers that cover the known V regions found in *TCR*γ rearrangements. The assignation of individual primers to mix 1 or mix 2 was initially arbitrary but experience has shown that mix 1 will amplify the majority of rearrangements. Mix 1 contains V-region primers 2–4, 8, and 9, each at 100 ng/µL (total 500 ng/µL). Mix 2 contains V-region primers 5, 10–12, each at 100 ng/µL (total 400 ng/µL). Either mix 1 or mix 2 is used in combination with mix 3. Mix 3 is a mixture of three primers shown as follows, each at 100 ng/µL (total 300 ng/µL), which cover the five J regions known to be involved in T-cell rearrangements. These primers are used in all PCR reactions for the detection of clonal rearrangements.

   V2 5′ CTT CCT GCA GAT GAC TCC TAC AAC TCC AAG GTT G 3′
   V3 5′ CTT CCT GCA GAT GAC GTC TCC ACC GCA AGG GAT G 3′
   V4 5′ CTT CCT GCA GAT GAC TCC TAC ACC TCC AGC GTT G 3′
   V5 5′ TTC CTG CAG ATG ACG TCT CCA ACT CAA AGG ATG 3′
   V8 5′ CTT CCT GCA GAT GAC TCC TAC AAC TCC AGG GTT G 3′
   V9 5′ GG(A/G/C/T) ACT GCA GGA AAG GAA TCT GGC ATT CCG 3′
   V10 5′ CTC TGC AGA ATC CGC AGC TCG ACG CAG CA 3′
   V11 5′ CAC TGC AGG CTC AAG ATT GCT CAG GTG GG 3′
   V12 5′ ACT CTG CAG CCT CTT GGG CAC TGC TCT AAA 3′
   Segment J 1.3 and J 2.3: 5′ AAG TGT TGT TCC ACT GCC AAA 3′
   J 1.1 and J 2.1: 5′ AGT TAC TAT GAG C (C/T)T AGT CCC 3′
   J 1.2: 5′ TGT AAT GAT AAG CTT TGT TCC 3′

5. Sterile mineral oil.
6. Controls.
   a. Monoclonal T cells: T-lymphocyte clones that generate a monoclonal band with mix 1/3 or mix 2/3. Adjust DNA to 100 ng/µL. Use 100 ng per reaction.
   b. Monoclonal B-cell line U266 ***(28)***: Adjust DNA to 100 ng/µL and use 100 ng per reaction. The PCR product will be 96 bp.
   c. Polyclonal normal peripheral blood DNA: Adjust DNA to 200 ng/µL. Use 200 ng per reaction.

      The primers, monoclonal and polyclonal DNA controls and buffer solutions should be frozen in aliquots at –70°C and used only once to prevent contamination. After thawing, vortex and pulse spin before use.
7. Omnigene Thermocycler (Hybaid, Middlesex, UK).

2.3. Polyacrylamide Gel Electrophoresis of PCR Products

1. Acrylamide bisacrylamide stock (30%) (Pharmacia Biotech, Uppsala, Sweden).
2. Tris borate ethylenediaminetetraacetic acid (EDTA) buffer (TBE) stock 5X: 0.45 *M* Tris-borate, 0.01 *M* Na_2EDTA.
3. Tetramethylethylenediamine. (TEMED)
4. Ammonium persulphate (40%).
5. Ficoll loading dye 6X: 0.25% bromophenol blue, 15% Ficoll (Pharmacia Type 400) in water.
6. Molecular weight markers, 20 bp markers (Gibco-BRL, Life Technologies, Grand Island, NY).
7. Ethidium bromide stock 1%.
8. Bethesda Research laboratories (BRL) vertical electrophoresis system, No. 1071, Model 16-2.
9. Long wavelength UV light source (360 nm)

3. Methods

3.1. DNA Extraction Methods

The method of choice for DNA extraction will be dependent on the type of sample. Fresh samples should be used where possible. Historically, we have used standard methods of phenol extraction and ethanol precipitation for larger samples that give the purest DNA. However, these methods are time consuming, use toxic agents, and may not give good recovery for very small samples. Listed as follows are our methods of choice that we use for extraction of different sample types based on reproducibility and speed. Standard phenol extraction could be used as an alternative to Wizard Kit extraction for peripheral blood, bone marrow aspirates and trephines, lymph node biopsies, and larger samples of pleural effusions. In general, half of the sample is processed for DNA extraction and the other half is stored at –70°C for future use if required.

3.1.1. Bone Marrow Aspirates and Peripheral Blood

Samples can be left at 4°C for up to 3 d or frozen at –70°C before processing. This method is exactly as outlined in the instruction pamphlet that accompanies the Wizard DNA purification Kit (Promega Biotech). The manufacturer suggests that the DNA yield is 5–15 µg/300 µL of whole blood. This equates to $1.2–3 \times 10^6$ cells for normal peripheral blood, which needs to be taken into account when extracting DNA from blood or bone marrow samples with high white cell counts.

3.1.2. Lymph Node Biopsies

Lymph node biopsies are macerated using scalpel blades to ensure that the tissue is homogeneous. Half of the sample is then stored as a backup at –70°C and then the other half is extracted using the Wizard Kit as earlier.

3.1.3. Lymph Node Aspirates and Pleural Effusions ***(20)***

Samples are best handled on the day of receipt but can be stored at 4°C overnight.

1. Microfuge cells at 3300*g* for 2 min. If a pleural effusion is viscose then centrifuge at 12,400*g* for 5 min to ensure that all cells are deposited in the pellet.
2. Resuspend cells in 1 mL sterile saline and perform a manual nucleated cell count using a haemocytometer (Improved Neubauer). Divide sample into two tubes.
3. Microfuge at 3300*g* for 2 min.
4. Remove supernatant (*see* **Note 3**). Store one pellet at –70°C as a backup sample.
5. Add extraction buffer containing 6 µL proteinase K (1 mg/mL)/100 µL extraction buffer to the pellet to make a final concentration of $1–5 \times 10^4$ cells/µL with a minimum volume of 25 µL.
6. Incubate at 58–60°C for 1 h.
7. Incubate at 95°C for 10 min to inactivate Proteinase K.
8. Store at –70°C until ready to set up PCR.

3.1.4. Paraffin sections ***(30)***

1. Place 2–3 10 µm paraffin sections into 800 µL xylene to dissolve the paraffin.
2. Vortex vigorously for 10 s.
3. Spin at 12,400*g* for 5 min in a microfuge.
4. Discard supernatant.
5. Add 800 µL 100% ethanol.
6. Vortex vigorously and spin as before.
7. Repeat **steps 4–6**. Remove as much ethanol as possible.
8. Dry tissue pellet in a vacuum centrifuge for 2 min or until just dry.
9. Add 200–400 µL of extraction buffer depending on the amount of tissue visible in the paraffin sections. Add 6 µL proteinase K (1 mg/mL)/100 µL of extraction buffer.
10. Incubate for 60 min at 58–60°C for 1 h to overnight (longer times are usually better).
11. Incubate for 10 min at 95°C to inactivate proteinase K.
12. Store at –70°C until the PCR is performed.

3.1.5. Bone Marrow Trephines

The sample may be left at 4°C overnight before processing.

1. Transfer the bone marrow trephine transport liquid to a centrifuge tube and the trephine core to a sterile Petri dish containing 3 mL K_2 EDTA (1 mg/mL in saline).
2. Using disposable scalpel blades, cut up/crush/scrape the trephine to free the cells. Disperse the free cells by repeated drawing into a 2-mL syringe without a needle.
3. Transfer the solution to the bone marrow trephine transport liquid.
4. Spin 500*g* for 5 min.
5. Resuspend cells in 10 mL sterile saline.
6. Spin 500*g* for 5 min.
7. Remove supernatant.

8. Resuspend cells in 1 mL sterile saline and perform a manual nucleated cell count using a haemocytometer (Improved Neubauer).
9. Extract DNA using a Wizard DNA purification kit.

3.2. PCR

Extreme caution must be taken to ensure that PCR products from previous reactions do not contaminate samples. Pre- and post-PCR procedures and equipment are geographically separated, all solutions are stored as aliquots and only used once, and the precautions outlined by Kwok and Higuchi ***(31)*** are strictly followed.

3.2.1. DNA Samples

All patient samples must be tested in duplicate (*see* **Note 4**).

1. Use approximately 100–200 ng of DNA for *IgH* or *TCR*γ PCR's.
2. Use 10 µL or less of DNA extracts from paraffin sections.
3. Use 5 µL or less of DNA extracts from lymph node aspirate (LNA) samples where possible.
4. Controls. Each method of extraction requires the processing of a negative control for each batch of specimens. These should be tested by PCR at the same time as the samples under examination. Also include a control of 10 µL of water in every PCR experiment. Controls from known monoclonal cell lines and polyclonal (normal peripheral blood) DNA's must also be included in every PCR experiment.

3.2.2. PCR Protocol for Detection of IgH Gene Rearrangement

1. Make the following PCR stock primer mix for $n + 1$ tubes (n = total number of samples + positive + negative controls.

	µL/tube	Final amount
5X buffer	5.0	1X
FR3A (100 ng/µL)	1.0	100 ng
VLJH (100 ng/µL)	1.0	100 ng
AmpliTaq Gold™ (5 U/µL)	0.1	0.5 U
Water	7.9	

2. Add 15 µL of stock mix to sterile 0.5 mL polypropylene microtubes (Sarstedt, Australia).
3. Add 25 µL sterile mineral oil to each tube.
4. Add DNA to each tube (below oil) to give a final 25 µL PCR.
5. Cap tubes and label.
6. Mix and pulse spin (2 s) tubes.
7. Place in PCR machine.

PCR file conditions: Denaturation and activation of AmpliTaq Gold 94°C 15 min, then 45 cycles of: annealing at 60°C for 1 min, extension at 72°C for 1 min, and denaturation at 94°C for 1 min. This is followed by 60°C for 1 min and a 20-min extension at 72°C.

3.2.3. PCR Protocol for Detecting TCRγ *Gene Rearrangements*

1. Make the following PCR stock primer mixes for n + 1 tubes. (n = total number of samples + positive + negative controls).

TCR Mixes 1 and 3

	µL/tube	Final amount
5X buffer	5.0	1X
Primer mix 1 (500 ng/µL)	1.0	100 ng/primer
Primer mix 3 (300 ng/µL)	1.0	100 ng/primer
AmpliTaq Gold (5 U/µL)	0.1	0.5 U
Water	7.9	

TCR Mixes 2 and 3

	µL/tube	Final amount
5X buffer	5.0	1X
primer mix 2 (400 ng/µL)	1.0	100 ng/primer
primer mix 3 (300 ng/µL)	1.0	100 ng/primer
AmpliTaq Gold (5U/µL)	0.1	0.5 U
Water	7.9	

2. Add 15 µL of stock mix to sterile 0.5 mL polypropylene microtubes (Sarstedt, Australia).
3. Add 25 µL sterile mineral oil to each tube.
4. Add DNA to each tube (below oil) to give a final 25 µL PCR.
5. Vortex mix and pulse spin (2 s).
6. Place tubes in PCR machine.

   PCR file conditions (*see* **Note 5**): Denaturation and activation of AmpliTaq Gold™ 94°C × 15 min, then 35 cycles of annealing at 63°C for 1 min, extension at 72°C for 1 min, and denaturation at 91°C for 1 min. This was followed by 63°C for 1 min and a 20-min extension at 72°C.

3.3. Electrophoresis of PCR Products (see Note 6*)*

1. Prepare a 6% polyacrylamide gel (30 mL for 19 × 20 cm): Add 6 mL 30% stock 1.5 : 30 bisacryl : acrylamide to 3 mL 5X TBE and make up to 30 mL with water in a conical flask. Add 30 µL TEMED and 60 µL ammonium persulphate (40%).

Mix and pour immediately between glass plates, which are separated by 1 mm spacers and position well comb. Leave to set for 1 h. CAUTION: unpolymerized acrylamide is a known neurotoxin.

2. Load 7 µL of the PCR product mixed with 4 µL Ficoll loading dye and electrophorese at 250 V until the dye front is 2 cm from the end of the gel for IgH and the end of the gel for TCRγ.
   Include molecular weight DNA markers in one lane.
3. Stain the gel by removing one plate and immersing gel in 0.5 µg/mL ethidium bromide in 0.5X TBE solution for 10 min. Remove gel and visualize/photograph under long-wavelength UV light (360 nm). CAUTION: ethidium bromide is a known mutagen.

3.4. Interpretation of Polyacrylamide Gels

Normal samples will give PCR products of varying lengths and are termed polyclonal (P). IgH PCR products range from 70–130 bp and TCRγ PCR products range from 170–230 bp. A discrete PCR product will be detected in a malignant sample representing either the *IgH* or the *TCR*γ rearrangement of the malignant clone. These samples are termed monoclonal (M). Sometimes two or more discrete *IgH* or *TCR*γ gene rearrangements are observed in a patient sample. These could be due to the presence of more than one malignant clone or because both *IgH* or *TCR*γ genes in the same clone have rearranged. These samples are designated as monoclonal with the number of observed bands placed in parentheses, e.g., M(2).

For some specimens the results will be very clearly P or M. Often a monoclonal product will be very clearly seen against a polyclonal background (M + P). For others, interpretation may be more difficult (*see* **Notes 7** and **8**). If there is any doubt, then the PCR should be repeated in duplicate and the new PCR products electrophoresed alongside the duplicate products from the previous test. If a band is detected at the same position in all samples, then it is likely that a monoclonal band is present, *see* **Figs. 1** and **2**.

3.5. Clinical Significance

3.5.1. Clonality detection

1. A monoclonal *IgH* or *TCR*γ rearrangement is indicative of a malignant population of cells. Some B-cell malignancies also possess a *TCR*γ rearrangement. Malignancies of T-cell origin very rarely also have an *IgH* rearrangement.
2. A polyclonal result will indicate one of three possibilities:
   a. No malignant cells are present in the sample.
   b. A malignant population is present but the primers used in our method can not detect it.
   c. A malignant population is present but it is present at levels below the limit of detection of our test (approx 5–10% in PB and LN, 0.5–5% in BM).

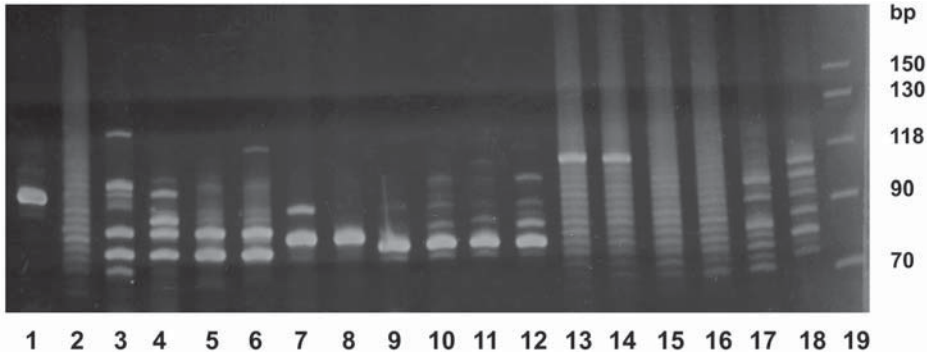

Fig. 1. Detection of *IgH* monoclonality in different sample types. Samples were amplified using PCR and the *IgH* PCR products analyzed by electrophoresis on 6% polyacrylamide gels. M = monoclonal, (M2) = monoclonal with two bands, P = polyclonal, M + P = monoclonal against a polyclonal background. **(Lane 1):** U266 monoclonal control (M). **(Lane 2):** Normal peripheral blood polyclonal control (P). **(Lanes 3 and 4):** Bone marrow aspirate at diagnosis from patient 1 with B-cell acute lymphoblastic leukemia of childhood (M2). **(Lanes 5 and 6):** Bone marrow trephine after 2 wk intensive induction therapy (morphological remission) from patient 1 showing the same 2 bands as at diagnosis (M2). **(Lanes 7 and 8):** Fresh lung sample post-treatment for lymphoma (M). **(Lanes 9–12):** Lymph node aspirate, query NHL (M). **(Lanes 13 and 14):** Paraffin section of skin, query lymphocytoma (M + P). **(Lanes 15 and 16):** Lymph node, query lymphoma (P). **(Lanes 17 and 18):** Lymph node—query lymphoma (P but only few genomes amplified). **(Lane 19):** Molecular weight markers.

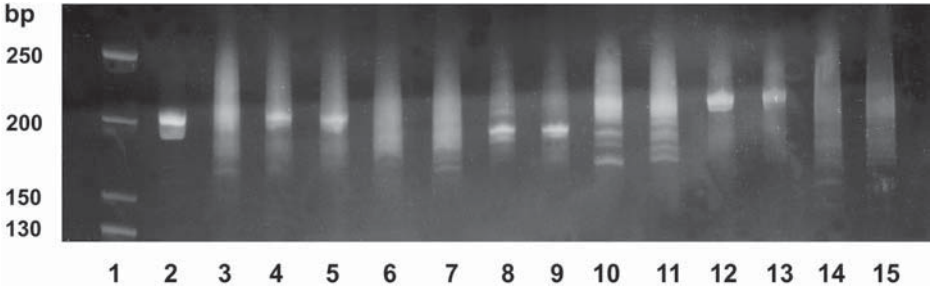

Fig. 2. Detection of *TCR*γ monoclonality in different sample types. Samples were amplified using PCR and the *TCR*γ PCR products analyzed by electrophoresis on 6% polyacrylamide gels. M = monoclonal, P = polyclonal, M + P = monoclonal against a polyclonal background. *TCR*γ primer mix1/3 was used in all cases except for lanes 6 and 7 where primer mix 2/3 was used. **Lane 1**: Molecular weight markers. **Lane 2**: T-cell monoclonal control (M). **Lane 3**: Normal peripheral blood polyclonal control (P). Lanes 4–7: fresh skin biopsy, M +P with primer mix 1/3, **lanes 4–5**, and P with primer mix 2/3, **lanes 6–7**. Lanes 8–9: Patient 1 (same as in Fig. 1) at diagnosis (M +P). **Lanes 10–11**: Patient 2, bone marrow trephine at day 35 (P). **Lanes 12–13**: peripheral blood, query LGL leukemia (M). **Lanes 14–15**: Ascitic fluid, query non-Hodgkin's lymphoma (P).

Approximately 75% of *IgH* monoclonal populations can be detected using the primers outlined here. The percentage detected will vary for different tumor types (*see* **Subheading 1.2.**). Most *TCR*γ monoclonal populations are detected by mix 1 and mix 3 primers and a lesser number with the mix 2 and mix 3 primer combination. Approximately 75% of *TCR*γ monoclonal populations can be detected if both mixes are used. Therefore a monoclonal result is more informative than a polyclonal result.

3.5.2. Residual and Metastatic Disease

The size of the *IgH* or *TCR*γ rearrangement can act as a molecular marker when monitoring disease throughout patient treatment. If a monoclonal PCR product is detected at e.g., d 35 of induction therapy in acute lymphoblastic leukemia of childhood, and it is a product of the same molecular weight as that found at diagnosis in that patient then this is evidence that a detectable amount of disease is still present in the patient. When studying residual disease samples, it is important to also include a sample taken at diagnosis from the same patient, in order to determine if the same size PCR product is detected. The size of the monoclonal PCR product also acts as a marker to identify if a malignancy has spread to other tissues.

4. Notes

1. It should be remembered that the PCR's described here involve consensus primers to perhaps many thousands of different but homologous gene rearrangement targets. Each sample will have a different repertoire of gene rearrangements whether they be normal or malignant. This means that the kinetics of the reaction for every PCR will be different. The consensus primers will be better matched for some of the gene rearrangements compared with others. This can lead to preferential amplification. If the gene rearrangement of the malignant clone is a poor match for the consensus primers, then even if it is present at high levels, the normal gene rearrangements that may have varying ability to bind the consensus primers can outcompete the malignant rearrangement. It is possible sometimes to overcome this preferential amplification by limit diluting the sample to a few genomes, thus removing the competition of large numbers of normal products. If the malignant clone is present more often than the other rearrangements, then the same size PCR product should appear in replicate amplifications of the dilutions.
2. Samples that do not show monoclonality at the *IgH* gene using the consensus primer FR3A described here may show monoclonality using the consensus primer FR2B ***(32)***. Samples that fail to show monoclonality using the consensus primers FR3A and FR2B may show monoclonality using primers in the VH4 and VH5 region. (i.e., VH4a, VH4b and VH5 ***(33)***.

3. If there is red blood cell contamination, then resuspend the pellet in 1 mL lysis buffer, vortex briefly, and centrifuge immediately at 3300*g* for 2 min to lyse the red cells (longer exposure to the buffer may disrupt the lymphocytes). Remove supernatant.
4. As the methods described here have the ability to detect a single IgH or TCRγ rearrangement, it is possible to get a monoclonal band that has arisen from just one molecule in a sample that has a limited number of genomes. This could possibly result in a false positive. It is therefore very important to perform all diagnostic tests for any sample in duplicate. If the population of cells is monoclonal then duplicate samples would give the same-size PCR product. For polyclonal populations, different-sized PCR products would result. For paraffin sections where the DNA is of poor quality or in lymph node aspirates where there are often low numbers of lymphoid cells, we routinely run samples in quadruplicate.
5. It is important to perform hot-start ***(34)*** PCR for the IgH PCR system. We find that AmpliTaq Gold provides a convenient hot-start method.
6. It is recommended that separation of PCR products be performed on polyacrylamide gels as outlined here because it provides better resolution than agarose for PCR products of this size range.
7. There are several general inhibitors of PCR that can potentially inhibit PCR for these tests. Hemoglobin will inhibit PCR and so it is important to lyse red blood cells to remove haemoglobin and phenol extraction will also achieve this. Heparin will inhibit PCR and so it is important to ensure that peripheral blood and bone marrow samples are sent in EDTA anticoagulant. If necessary, a heparanized sample can be treated with Heparinase II, prior to the PCR. Analysis of paraffin sections can be very difficult. The sample is often very small and the formalin treatment will irreparably damage the DNA. Therefore, if fresh samples are potentially available, as is often the case with lymph node biopsies, then these could be set aside at –20°C or –70°C and analyzed if necessary at a later date. If a sample does not give any PCR amplification products then dilution of the sample will sometimes result in dilution of inhibitors and a PCR product will be detected in an otherwise negative sample. This is particularly relevant for paraffin sections and aspirates where the DNA is prepared as a crude extract.
8. Sometimes a nonspecific PCR product appears at approximately 180 bp with mix 1 and mix 3 primers. This band is smaller than most TCRγ rearrangements. This is not usually a problem for interpretation as it often appears across the whole PCR experiment including in the polyclonal control, but it is important to keep this in mind. Occasionally, it is necessary to dilute the mix 1 and mix 3 PCR product 1/5 in order to see monoclonal bands clearly on the polyacrylamide gel. The reason for this difference in final amount of PCR product from experiment to experiment is not known.

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7

Quantifying Residual Leukemia by "Clone-Specific" Polymerase Chain Reaction

Michael J. Brisco

1. Introduction

Molecular biology tests can quantify extremely low levels of cancer cells, provided that a genetic marker for the cancer is known. Acute lymphoblastic leukemia (ALL) exhibits many genetic abnormalities, but most are uncommon or technically difficult to use as markers for sensitive quantification. We therefore use the leukemia clone's rearranged immunoglobulin heavy-chain (IgH) gene as a clonal marker ***(1,2)***. A very sensitive, quantitative polymerase chain reaction (PCR) test with "clone-specific" primers can be developed for 60–70% of B-lineage ALLs (*see* **Fig. 1**).

This method can quantify ALL at 10^{-5} (1 leukemia cell in 100,000 normal cells), and often at 10^{-6}, and has been used to study over 150 patients. The level of ALL in marrow at the end of induction treatment is an independent and powerful predictor of outcome ***(3–7)***. Long-term monitoring during remission can detect persisting leukemia, or provide early warning of relapse ***(8,9)***. Other methods use rearranged T-cell receptor γ or δ genes as markers, and different techniques for detection and quantification, which vary in simplicity, predictive power, and precision ***(10–15)***. ALL has been quantified in marrow, blood, and stem-cell harvests (e.g. 16). Other clonal B-cell disorders, such as multiple myeloma and non-Hodgkin's lymphoma, can also be studied (e.g. ***17,18***). For a recent review of minimal residual disease in childhood leukemia, see **ref. *19***.

Briefly, DNA is extracted from marrow taken at diagnosis. PCR with consensus primers amplifies a fragment of the leukemia clone's rearranged IgH gene. A large-scale PCR with M13 linker primers is used to synthesize enough product for automated direct sequencing. Using M13 primers in the sequencing reaction generates clearer sequence, and ensures that IgH sequence close to

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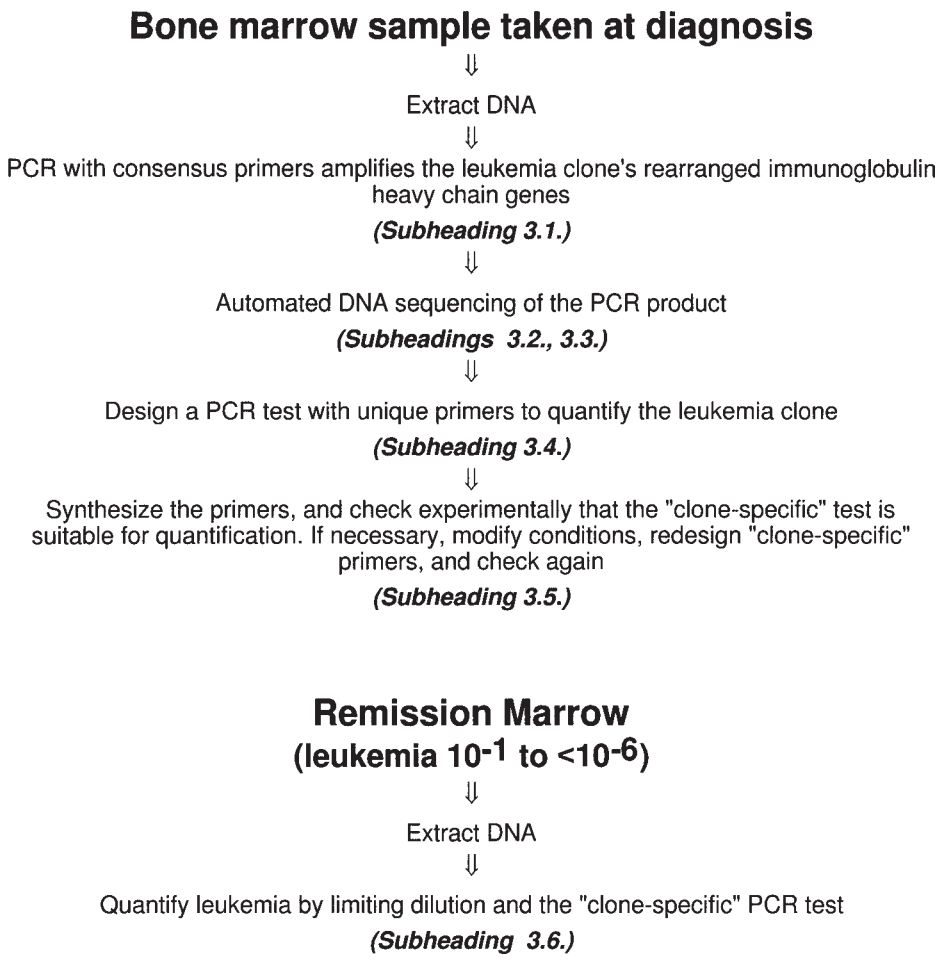

Fig. 1. Strategy for quantifying residual disease.

the sequencing primers can still be read. The resulting sequence is analyzed by computer, to identify its most variable parts, to which "clone-specific" PCR primers are designed. Thus, a unique "clone-specific" PCR test to quantify the leukemia clone is developed for each patient. Before use, the test must be shown to be specific, and quantify low levels of leukemia. The clone-specific primers are synthesized, DNA from marrow at diagnosis is diluted, and small amounts of this DNA, containing a few leukemia targets, are added to PCR reactions containing DNA from a large number of normal blood cells. The clone-specific PCR test should generate large amounts of product from one to

two molecules of the leukemia clone's rearranged IgH gene, but should not generate any product from other rearranged IgH genes from normal lymphocytes, or from other genes. It can then be used to quantify ALL in remission samples.

Limiting dilution PCR is well suited to quantifying low levels of leukemia in remission tissue samples ***(20)***. DNA extracted from tissue samples is diluted serially, and several aliquots of DNA at several different concentrations are amplified by PCR with many cycles, which give large amounts of product from as few as one to two target DNA molecules. At the limit of dilution, each PCR tube receives on average one target DNA molecule. However, due to random variation, a few tubes will receive two or more target molecules, and a few will receive none. PCR therefore yields either a large amount of product, or else no product at all, and tubes can be scored as positive or negative for amplification. The average number of PCR target molecules per tube can be calculated from the fraction of tubes that are negative for amplification, assuming that the data fit a Poisson distribution. The level of leukemia can then be calculated from the amount of DNA added to each tube, assuming each cell contains 6 pg DNA ***(21)***, and each leukemia cell contains one rearranged IgH gene target. A correction is applied for the proportion of DNA targets that are not amplifiable, which is assessed experimentally (*see* **Subheading 3.5.3.** and **Note 1**). The efficient PCR conditions make this technique ideal for quantifying low levels of leukemia, particularly in small samples. The presence or absence of PCR product is easily determined by gel electrophoresis. Competitive PCR, and quantification techniques that require a standard rate of amplification, may be less useful, because different patients have different PCR tests, requiring different competitor molecules, and with different amplification rates.

Quantification of residual disease in this way presents many challenges, and the method has to cope with variations without becoming too complicated.

1. Since rearranged IgH genes vary, two consensus primers are used, to obtain a marker sequence from most patients.
2. Some leukemia clones have several rearranged IgH genes, whose PCR products have to be separated, by electrophoresis or limiting dilution, before preparing DNA for sequencing. The product that is most clearly separate from the others is used as the clonal marker, because it usually gives the clearest sequence.
3. Automated sequencing is reliable, but a few PCR products produce sequencing data that is difficult to interpret. Causes include low DNA yield, chemical impurity, and contaminating sequences.
4. Because rearranged IgH sequences vary greatly, the best binding sites for clone-specific primers also vary. We therefore study each sequence in detail, to understand its structure, and then design primers accordingly.
5. However, some clone-specific primers turn out to amplify other genes when used for PCR. To keep quantification simple, we use one set of reaction conditions

with a few standard but robust PCR programs, and improve specificity by redesigning the primers. Since specificity depends on the sequences at their 3′ ends, adding or removing a few bases usually solves the problem.

6. Limiting dilution quantification requires 12–16 DNA concentrations per tissue sample, since levels of ALL can vary. Studying all DNA concentrations in a single experiment would waste reagents and DNA because only the concentrations around the limit of dilution provide useful information. We therefore test 5 or 6 DNA concentrations around the most likely limit of dilution, and then study any other concentrations needed to complete the data set.

2. Materials

2.1. Tissues and DNA Extraction

Fresh or frozen aspirated bone marrow is used most often, but trephines, and peripheral blood can also be studied. As a control for some experiments, peripheral blood from a healthy volunteer is required. DNA extraction is described by P. J. Sykes in this volume. DNA is resuspended in PCR-clean water at 0.5 μg/μL.

2.2. Precautions Against Contamination

Quantification depends on detecting small numbers of DNA molecules, and often uses nested PCR. Rigorous precautions against contamination, as described by P. J. Sykes in this volume, are vital. Three separate rooms are needed for PCR: one for extracting DNA and setting up reactions, one for electrophoresing PCR products, and one for diluting PCR product in clean conditions, and setting up reactions that use it as template. New IgH sequences should be compared to others previously sequenced in the same laboratory to confirm that they are not contaminants.

2.3. Primers

Consensus primers to the rearranged IgH gene (*see* **Fig. 2** on facing page).

1. LJH: 5′ TGAGGAGACGGTGACC 3′
2. ELJH: 5′ TGAGGAGACGGTGACCAGGATCCCTTGGCCCCAG 3′
3. FR2B: 5′ GTCCTGCAGGC(C/T)(C/T)CCGG(A/G)AA(A/G)(A/G)GTCTGGA GTGG 3′
4. FR3A: 5′ ACACGGC(C/T)(G/C)TGTATTACTGT 3′
5. M13 Linker primers: the 3′ end can bind to FR2B, FR3A, or ELJH; and M13 sequencing primers can bind to the 5′ end (*see* **Fig. 2**)
6. FR3A-link: 5′ ACGACGGCCAGT ACACGGC(C/T)(G/C)TGTAT 3′
7. FR2B-link: 5′ TGTAAAACGACGGCCAGT CCTGCAGGCCT 3′
8. ELJH-link: 5′ ATTTCACACAGGAAACAGCTATGAC CAGGATCCCT 3′

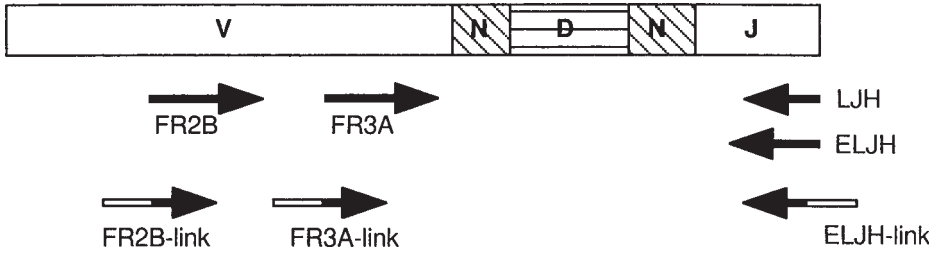

Fig. 2. Locations and orientations of consensus primers in a typical rearranged immunoglobulin heavy-chain gene. Primers FR2B and FR3A are in the framework 2 and 3 regions of V, respectively. ■: heavy-chain gene sequence; ❑: M13 sequence that sequencing primers can bind to. Arrows point from 5′ to 3′.

9. N-ras primers:
   a. N-ras 1: 5′ TGACTGAGTACAAACTGGTGG 3′
   b. N-ras 2: 5′ CTCTATGGTGGGATCATATTCA 3′
10. M13 Sequencing primers:
   a. V-seq: 5′ GTAAAACGACGGCCAGT 3′
   b. J-seq: 5′ ATTTCACACAGGAAACAGCTATGAC 3

Commercially synthesized primers (Geneworks, Adelaide, South Australia, or DNA Express, Fort Collins, CO) are redissolved according to manufacturers instructions, and no elaborate purification is required. For clone-specific primers, synthesis on a 40-nmol scale yields enough for quantifying leukemia in several tissue samples.

2.4. Conditions for the Polymerase Chain Reaction

PCR buffers are described by P. J. Sykes in this volume. PCRs usually have final volume 25 µL, containing 50 ng each primer, 0.2 units (U) native *Taq* polymerase (Biotechnology International, Perth, Australia) or 0.5 U Amplitaq Gold (Perkin-Elmer Applied Biosystems, Foster City, CA), and 2.0 µL template. Hot start ***(22)*** is often used to improve specificity by preventing priming and extension at low temperatures. Amplitaq Gold gives good results. However, much of the original quantification work was done as follows. Dispense the PCR primers in 10 µL water to the bottom of each PCR tube, and centrifuge briefly. Add a 15–20 µL pellet of paraffin wax (e.g., as used for embedding histology specimens). Use a heating block to melt the wax. Tap the tube to remove large air bubbles so that the molten wax floats on the primer mix. When the wax sets, it will seal the primer mix in the bottom of the tube. Add remaining 15 µL of PCR ingredients (including native *Taq* polymerase), then oil, and

Table 1
PCR Profiles

Program	Annealing	Extension	Melting	Cycles
A	55°C—10 s	72°C—15 s	91°C—10 s	45
B	60°C—10 s	72°C—30 s	92°C—10 s	45
C	25°C—30 s	45°C—60 s	94°C—30 s	10
	50°C—30 s	72°C—30 s	94°C—30 s	30
D	50°C—30 s	72°C—30 s	93°C—30 s	5
E	65°C—15 s	72°C—15 s	91°C—10 s	2
	64°C—15 s	72°C—15 s	91°C—10 s	2
	63°C—15 s	72°C—15 s	91°C—10 s	2
	62°C—15 s	72°C—15 s	91°C—10 s	2
	61°C—15 s	72°C—15 s	91°C—10 s	2
	59°C—15 s	72°C—15 s	91°C—10 s	2
	57°C—15 s	72°C—15 s	91°C—10 s	2
	55°C—15 s	72°C—30 s	93°C—15 s	30
F	55°C—10 s	74°C—30 s	95°C—10 s	60

centrifuge briefly. Template is added last, and the tube centrifuged to bring it through the oil layer into the PCR buffer.

2.5. Thermal Cycler and PCR Programs

Each PCR program consists of five parts: (1) 94°C—5 min for native *Taq*, 15 min for Amplitaq Gold; (2) one of the profiles listed in **Table 1**; (3) final annealing temperature—1 min; (4) 72°C—5 min; (5) cooling to room temperature. Most programs are used on a Hybaid Omnigene 3 Thermal Cycler (Hybaid, Teddington, UK). Program C needs extra cooling, and is run on a Cetus DNA Thermal Cycler.

2.6. Electrophoresis

Nondenaturing polyacrylamide gels, 6%: instructions, recipes, and conditions, including Tris-borate-EDTA (TBE) buffer, and loading buffer, are in the Chapter 6. Preparation of sequencing templates (*see* **Subheading 3.2.**) requires a gel containing 1.5–2.5% of 24–28°C gelling-point agarose (Progen, Darra, Queensland, Australia), 0.5 µg/mL ethidium bromide, in 0.5X TBE buffer, electrophoresed in 0.5X TBE buffer at 40–80 V.

2.7. Ethanol Precipitation of DNA

To the DNA solution in a 1.5-mL flip-top or screw-cap plastic tube, add 2.5 vol 100% ethanol, 0.1 vol 3 *M* sodium acetate pH 5.5, and precipitate the DNA

at –20°C, overnight. Centrifuge in a microfuge, 10 min, to pellet DNA. Remove the supernatant, wash the pellet with 70% ethanol, and dry it in a vacuum centrifuge.

2.8. DNA Samples for Sequencing

The gel slice containing the PCR product is placed in a 1.5-mL plastic tube with 500 µL of PCR-clean water, frozen and thawed three times, and stored for 7 days at 4°C. The eluate is transferred to a new tube, water removed by drying in a vacuum centrifuge, and the pellet is resuspend in 50 µL sterile water.

To estimate the concentration of a DNA sample for sequencing: make up 30 mL of a 1% agarose gel in 0.5% TBE buffer, containing 500 ng/mL ethidium bromide; pour into a Petri dish, leave to set and cool; for use, the gel's surface should be dry. As standards, use 0, 1, 2, 3, 4, 6, 8, and 10 ng/µL of DNA whose concentration is known accurately (e.g., herring sperm DNA; Promega Corporation, Madison, WI). Place a 3-µL drop of each standard, the undiluted sample, and a 1/10 dilution of the sample onto the gel, store the dish in the dark for 1–2 h, and then view with 360 nm UV light. Usually the 1-ng/µL DNA standard is just visible. Estimate DNA concentration by comparing the fluorescence of standards and sample.

2.9. DNA Sequencing

Dyedeoxy™ Terminator Cycle Sequencing Kits and an ABI 373A Automated Sequencer were used to sequence PCR product according to the manufacturer's instructions (Perkin-Elmer Applied Biosystems). Primer V seq is used to sequence from the V end of the rearranged IgH gene and J seq from the J end.

2.10. Principles of Quantification by Limiting Dilution PCR

Genomic DNA is first diluted to: 300 ng/µL, 100 ng/µL, 30 ng/µL, and so on, down to 0.3 pg/µL. The same diluted samples should be used for all quantification experiments. The limit of dilution for the PCR target (the *N-ras* gene or the leukemic IgH marker gene) is estimated, several consecutive DNA concentrations around it are selected, and five aliquots of each are amplified. Following PCR and electrophoresis, tubes are scored as positive or negative for amplification according to whether they contained PCR product of the predicted size. At the concentration of DNA where two or three of the five tubes give product, the presence of *large* amounts of product in positive tubes confirms efficient amplification from one to two target molecules. If this is seen and other controls behave as expected, then the concentration of PCR target can be estimated.

Statistical analysis requires data on DNA concentrations between, and including, the lowest concentration where all tubes gave PCR product, and the highest concentration where no tubes gave PCR product, with at least one other concentration in between. If the first experiment does not produce this, other DNA concentrations can be tested, and the data can be pooled. For each DNA concentration, the total number of aliquots studied, the number that were negative for amplification, and the mass of DNA (in picograms) per aliquot, are entered into a computer program which fits a Poisson distribution by the criterion of least χ^2 ***(23)***. A probability >0.05 suggests that the fit, and the estimated mean number of amplifiable PCR targets per picograms DNA, are acceptable.

2.11. Distinguishing Specific and Nonspecific PCR Products

For quantification, following electrophoresis, each band must be carefully classified as either a specific PCR product or a nonspecific product. A product compared to another several lanes away, or to DNA size markers, might appear to be of the size expected for the specific product, but in fact could be several bases larger or smaller. DNA size markers and one to two positive controls per gel can identify PCR products that are obviously nonspecific. PCR products about the size of specific product need to be checked accurately, ideally against a positive control in the next lane.

3. Methods

3.1. Assessment of the Leukemia Clone's Rearranged IgH Genes

1. For each patient, two aliquots, 2 µL each, of DNA from marrow at diagnosis are amplified in a two-round PCR (*see* **Table 2**). 2.5 µL of product from round I is diluted in 250 µL PCR-clean water, for use as a template for round II.
2. Dilute 2.5 µL round II PCR product in 250 µL PCR-clean water to prepare DNA for direct sequencing (*see* **Subheading 3.2.**). Analyze the remaining product by electrophoresis.
3. Following electrophoresis, the PCR products from a patient's leukemia cells appear as one, two, or up to five discrete bands, and both aliquots of genomic DNA should produce the same pattern. Different patients produce different patterns.
   a. Tubes 1–4: FR3A-ELJH products are between 90 and 140 base pairs (bp).
   b. Tubes 7–10: FR2B-ELJH products are between 240 and 300 bp (*see* **Note 2**).
   c. Tubes 5, 6, 11, and 12: no IgH-sized product, confirming no contamination.
4. Choice of material for preparing PCR product for direct sequencing (*see* **Subheading 3.2.**): Other things being equal, sequence FR2B-ELJH products in preference to the shorter FR3A-ELJH products.
   a. If there is only one leukemia IgH product from the patient, use the diluted round II material from **step 2**.

Table 2
PCR to Amplify Rearranged IgH Genes from Leukemia Cells

		Round I		Round II	
Tubes	Template	Primers	Program	Primers	Program
1,2	Patient DNA, 25 ng/µL	FR3A, LJH	A	FR3A, ELJH	A, hot start
3,4	Positive control DNA,[a] 25 ng/µL	FR3A, LJH	A	FR3A, ELJH	A, hot start
5,6	PCR-clean water	FR3A, LJH	A	FR3A, ELJH	A, hot start
7,8	Patient DNA, 25ng/µL	FR2B, LJH	B	FR2B, ELJH	B, hot start
9,10	Positive control DNA,[a] 25 ng/µL	FR2B, LJH	B	FR2B, ELJH	B, hot start
11,12	PCR-clean water	FR2B, LJH	B	FR2B, ELJH	B, hot start

[a]From a previously studied patient or cell line.

b. If there are >1 leukemia IgH products, and one is well separated from the rest, use a clean scalpel blade to cut out a small block of gel containing it; soak the block in 500 µL PCR-clean water overnight; use the eluate.
c. If all leukemia IgH products are very close together, further work is needed (*see* **Note 3**).
d. If the IgH products form a continuous smear or many bands (*see* **Note 4**), or if the replicates show different bands (*see* **Note 5**), sequencing is unlikely to succeed.

3.2. Preparation of PCR Products for Direct Sequencing

Two PCR products are prepared for each patient, one from each original aliquot of genomic DNA.

1. PCR templates and primers are listed in **Table 3**. The primers should match those used in **Subheading 3.1.** (to amplify the product chosen for sequencing).
2. After round I, electrophorese 7 µL PCR product to check that the linker PCR has worked. The product should be 37 or 43 bp longer than the template.
3. Round II: add 100 µL complete PCR mix including *Taq* and primers to the remaining 18 µL of round I product, and perform PCR on the same tube.
4. After round II, remove 100 µl of the aqueous phase to a new tube, precipitate the DNA (*see* **Subheading 2.7.**), and resuspend it in 15 µl gel loading buffer (*see* **Subheading 2.6.**).
5. Prepare a gel of low gelling temperature agarose (*see* **Subheading 2.6.**); load 1-2µl of the sample in one lane (to separate DNA samples, and for photography) and the rest in the adjacent lane (for sequencing).
6. After electrophoresis, view the gel under long wavelength UV light (360 nm). Product should form a single very bright broad band. Since UV light can damage DNA, making it difficult to sequence, use a sterile scalpel blade to cut out quickly the major band for each PCR product that is to be sequenced, leaving behind the

Table 3
PCR to Prepare Amplified IgH Gene DNA for Direct Sequencing

		Round I		Round II	
Tubes	Template	Primers	Program	Primers	Program
1,2	Diluted or eluted product from **Subheading 3.1., step 4**	J-link and FR3A-link or FR2B-link	C	same as round I	D
3,4	PCR-clean water				

narrower band of product in the next lane. Place the gel block in a 1.5-mL plastic tube, and then, photograph the gel.

7. Elute and quantify DNA (*see* **Subheading 2.8.**). Usual yield: about 500 ng DNA. (For lower yields, *see* Note 6.)

3.3. DNA Sequencing

For each patient, primer V seq is used to sequence the sense (+) strand of DNA sample 1, and primer J seq to sequence the antisense (–) strand of DNA sample 2, as described in **Subheading 2.9.** Their sequences should be complementary, confirming that they belong to the leukemia clone. SeqEd software (Perkin-Elmer Applied Biosystems) is used to resolve any differences, which are often due to sequencing artefacts. The final sequence is recorded as the coding (+) strand, in the direction 5′ to 3′. **Figure 2** exemplifies a typical FR3A-ELJH sequence. To interpret sequence data, *see* **Notes 7–11**.

3.4. Design of Clone-Specific PCR Primers

3.4.1 Identifying the Most Variable Parts of the Rearranged IgH Genes

Identification assists in designing specific primers and confirms that the sequence is a rearranged IgH gene. Complementarity determining region 2 (CDR2) is identified by its position from primer FR2B's binding site. In CDR3, stretches of random nucleotides (N regions) are located. MacVector software (Eastman Kodak Scientific Imaging Systems, New Haven, CT), or similar, is used to analyze sequences. To find the N-region nucleotides, identify V, D, and J (variable, diversity, and joining) segments, by comparing the rearranged IgH sequence to sequences of the unrearranged germline V, D, and J segments (for rapid comparison, *see* **Note 12**). A mismatch of greater than two consecu-

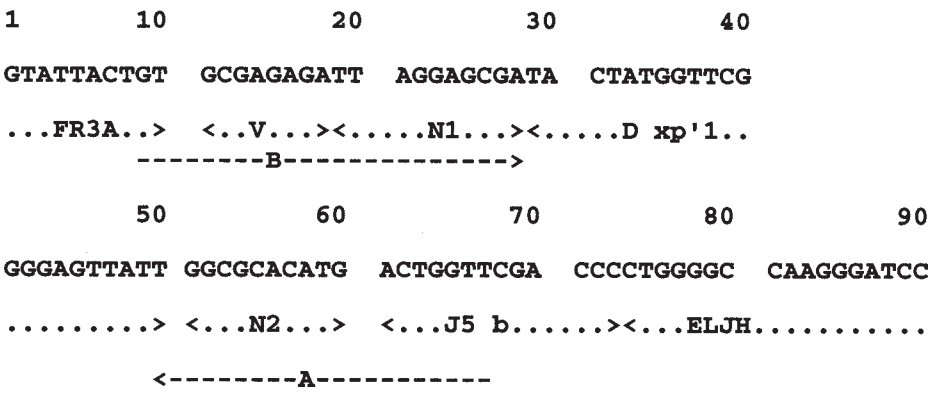

Fig. 3. Example of the DNA sequence from a rearranged immunoglobulin heavy-chain gene from a leukemia clone, showing: part of the binding sites for consensus primers FR3A and ELJH; the 3′ end of the V segment; a D segment, Dxp′1 ***(28)***; a J segment, J5b ***(29)***; N regions N1 and N2; and clone-specific primers A and B used for limiting dilution quantification.

tive bases indicates the end of homology. If several germline D (or J) segments show homology to the same part of the rearranged IgH gene, the segment with the longest continuous homology is recorded. **Figure 3** shows an example of a sequence with primer binding sites and the V, D, J, and N segments identified.

1. FR2B-ELJH sequences only: identify the FR2B primer's binding site.
2. FR2B-ELJH sequences only: identify CDR 2 approximately by numbering the 3′ G of FR2B as 1, and locating bases 8–58 to the right of this ***(24)***.
3. Identify the FR3A primer or its binding site. (If difficult to find, *see* **Note 13**.)
4. Identify the 3′ end of the V segment, which includes up to 8 bases of the consensus GCGA(G/A)A(G/A)A, immediately after the 3′ T of the FR3A primer's sequence. Follow any homology as far as possible to the right. (If there is no homology, *see* **Note 13**.)
5. Identify the ELJH primer's binding site.
6. Identify the 5′ end of the J segment, to the left of the ELJH primer binding site. (If there is no homology, *see* **Note 14**.)
7. Identify any D segments between V and J.
8. Identify N regions: these are unassigned sequence between V and J, and are often GC rich.
9. Expected result: most sequences turn out to be V-N_1-D-N_2-J rearrangements, but V-N-D-J, V-D-N-J, V-D-J, V-N-J, and, rarely, V-N_1-D_1-N_2-D_2-N_3-J are also seen. From 148 sequences, segment lengths (mean ± standard deviation) were: N_1, 10 ± 8 bp; D, 16 ± 11 bp; N_2, 6 ± 6 bp; J, 13 ± 7 bp.

10. For future reference, print out the IgH sequence showing the locations of segments and primer binding sites.

3.4.2. Design of PCR Primers for Quantification by Limiting Dilution PCR

The clone-specific PCR primers should be as specific as possible for their target sequence. They must also amplify their target efficiently, and their product must be clearly identifiable following electrophoresis. Achieving maximum specificity depends on the type and length of VDJ rearrangement. A pair of primers in CDR3, on opposite sides of D, and with N region sequence at their 3′ ends, often works. Using a total of 12–15 bases of the N region gives an average chance of success, and the chance increases somewhat if more N is used. Primer binding sites should be at least 10 bp apart so that leukemia-size products can be distinguished from short nonspecific products or primer dimers. A third rule is to avoid the problem of primer dimers. With a little ingenuity, a pair of primers in CDR3 can usually be designed that satisfies the above, or reaches a reasonable compromise between the need for a gap between primers, and including enough N-region nucleotides for specificity. However, for very short rearranged IgH sequences, it is better to design one primer in CDR2 and the other spanning CDR3. The steps set out as follows are a guide to primer design. Both strategies may need to be explored, and the principles applied with common sense. **Figure 3** shows an example of a CDR3 primer pair.

1. Decide which strategy to explore first:
   a. V-N-D-N-J or V-N-J with more than 15 bases between V and J; V-N-D-J; V-D-N-J: V-N-D1-N-D2-N-J: a pair of primers in CDR3—go to step 2.
   b. V-N-D-N-J or V-N-J with 15 or fewer bases between V and J: one primer in CDR2 and the other spanning CDR3—go to **step 3**.
2. Designing a pair of primers in CDR3:
   a. V-N-D-N-J rearrangements: design primer A's 3′ end in the shorter N region, as if to amplify the D segment during PCR. Primer B's 3′ end will be located in the longer N, on the opposite side of D, and >9 bases from primer A's 3′ end. Primer B's 3′ end should include as much of N as possible.
   b. V-N-D-J and V-D-N-J rearrangements: primer A's 3′ end is at the V-D or D-J boundary, and includes 3–4 bases of D; primer B's 3′ end should be in N.
   c. V-N-D-N-D-N-J rearrangements: design primer A in the second-longest N region and primer B in the longest N region.
   d. Long V-N-J rearrangements: design primers to amplify the middle 10 bases of N.
3. Designing a CDR2-CDR3 primer pair:
   a. CDR2 primer: the 3′ end should be at or near base 27 (*see* **Subheading 3.4.1., step 2**), corresponding to a highly variable part of CDR2 ***(24)***.

b. CDR3 primer: the 3′ end should be as variable as possible: placed next to the last base of V and including all of CDR3; or including the longest N segment.

4. All primer pairs: Avoiding primer dimers. If all of the 3′ end of one primer molecule can hybridize exactly to the 3′ end of another molecule, even if only by 2–3 bases, *Taq* polymerase might extend each primer, using the other as the template. The resulting short PCR product, a "primer dimer," is amplified very efficiently, reducing the yield of specific product. To avoid this problem, check the last 2 bases at the 3′ ends for possible hybridization either to the primer itself, or to another with which it would be used. Then check the last 3 bases; and so on up to the last 6 bases. If primers can hybridize exactly at their 3′ ends, redesign one or both, by adding a base to one 3′ end, and check again. Up to 3–4 bases of D can be added, provided that the primers remain >9 bp apart. For example: primer A in **Fig. 3** originally had its 3′ base designed to bind to G_{52}; however the primer's 3′ end . . . TGCGC could hybridize to itself. The primer was extended by 2 bases so that the 3′ end now binds to T_{50}, and the new 3′ endq.q.q. TGCGCCA is less likely to form dimers.
5. To design 5′ ends, aim for a melting temperature of 60–62°C, assuming that each A or T residue contributes 2°C, and each G or C residue contributes 4°C.
6. For future reference, print out the IgH sequence and record the location and orientation of clone-specific primers, and the expected size of products and possible primer dimers.

3.5. Testing "Clone-Specific" Primers

These two-round PCR experiments should confirm that the clone-specific PCR test is suitable for limiting dilution quantification. The primers should generate large amounts of leukemia-size product from one to two DNA targets mixed with DNA from normal cells (e.g., marrow or blood); and most or all leukemia cells should have the marker gene.

3.5.1. Amplification of Normal Peripheral Blood DNA for Rapidly Testing the Specificity of Clone-Specific Primers

This is performed as in section 3.1, except that DNA (500 ng/μL) from peripheral blood from a healthy volunteer is used instead of patient DNA, and its final PCR product should be a smear or ladder of bands (*see* **Notes 4 and 5**). The diluted first round products are used to test primer specificity (see below).

3.5.2. Initial Rapid Test of Specificity

This one-round PCR should confirm that the primers work, and are reasonably specific. If clone-specific primers fail, it is usually because they amplify nonspecific products. The most effective solutions are to try other primers, or alter PCR conditions (*see* **Note 15**).

Table 4
PCR Templates for Checking That "Clone-Specific" Primers Work and Are Reasonably Specific

Tubes	Template
1,2	Preamplified normal blood DNA from **Subheading 3.5.1.**
3,4	DNA sample sequenced, diluted 1:250
5,6	PCR-clean water

1. Use clone-specific primers with hot start to amplify the templates listed in **Table 4**. Use program E for leukemia-sized product <150 bp and program B for leukemia-size product >150 bp.
2. Analyze PCR products by electrophoresis. Expected results:
   a. Tubes 1 and 2: no leukemia-sized product, confirming specificity (if it contains several bands, or leukemia-sized product, *see* **Note 15**).
   b. Tubes 3 and 4: single strong band of leukemia-sized product (if absent, *see* **Note 16**).
   c. Tubes 5 and 6: no leukemia-sized product, confirming freedom from contamination.

3.5.3 Assessing the Proportion of PCR Targets That Are Amplifiable in the Diagnostic DNA Sample (see **Note 1***)*

Attempt amplification of a 110-bp fragment of the single copy *N-ras* gene, with PCR conditions that give large amounts of product from one to two target DNA molecules ***(20)***. The result is used to select DNA concentrations for further study.

1. Prepare a dilution series from the diagnostic DNA (*see* **Subheading 2.10.**).
2. Perform a single round hot start PCR (*see* **Table 5**) with primers *N-ras* 1 and *N-ras* 2 and program F:
3. Analyze products by electrophoresis. Expected results are as follows:
   a. Tubes 1–5: at least two yield strong 110 bp product, confirming efficient amplification.
   b. Tubes 6–35: working from high to low DNA concentration, the proportion of tubes with 110 bp product falls from 5/5 to 0/5; *and* the lowest DNA concentration to give the 110 bp product still gives *large* amounts of product.
   c. Tubes 36–40 (negative controls): no 110 bp product.
   d. For results other than these, *see* **Notes 17–20**.
4. Score tubes as positive or negative for the 110 bp product, and use Poisson statistics (*see* **Subheading 2.10.** and **2.11.**) to estimate the average number of amplifiable *N-ras* targets per 3 pg DNA (i.e., per haploid genome). This indicates what fraction of DNA targets can be amplified. Expected results: 0.2–1 amplifiable *N-ras* targets per 3 pg of DNA, but a few samples might contain a lower concentration of amplifiable targets.

Table 5
PCR to Assess the Proportion of *N-ras* PCR Targets That Are Amplifiable in a DNA Sample

Tubes	Template
1–5	Positive control DNA[a] with ≈0.5 amplifiable *N-ras* targets/µL
6–10	Sample DNA, 100 pg/µL
11–15	Sample DNA, 30 pg/µL
16–20	Sample DNA, 10 pg/µL
21–25	Sample DNA, 3 pg/µL
26–30	Sample DNA, 1 pg/µL
31–35	Sample DNA, 0.3 pg/µL
36–40	PCR-clean water

[a]If no previously studied sample is available, tubes 6–35 act as their own positive controls by fulfilling the expected results.

3.5.4. Testing Whether the Clone-Specific PCR Is Suitable for Limiting Dilution Quantification

Limiting dilution quantification is performed on diluted diagnostic DNA, and the level of amplifiable leukemia IgH targets observed is compared to the level expected from the amount of DNA used in PCR. Detection of one to two targets (6–12 pg DNA) is expected, but the result can be affected by random variation, whether all the DNA molecules can be amplified, whether all cells in the tissue were leukemia, whether all leukemia cells have the marker sequence, and whether the clone-specific primers work. The experiment differs from limiting dilution quantification in test samples (*see* **Subheading 3.6.**) as follows. Here, the limit of dilution is predictable, whereas in a test sample, a separate experiment (*see* **Subheading 3.6.2.**) is needed to find it. Here, the correction for amplifiability is applied at this stage, whereas for test samples, the correction is applied at the end of quantification (*see* **Subheading 3.6.3., step 4**). Finally, test samples may contain normal lymphocytes, whose rearranged IgH genes affect PCR efficiency ***(2)***, and here, a similar background is created by adding DNA from normal blood.

1. The templates are from the dilution series prepared previously, with concentrations close to those listed (*see* **Table 6**). In diagnostic marrow, each diploid genome should contain two copies of the *N-ras* gene and one copy of the rearranged IgH marker gene. Thus, a sample with 20 amplifiable *N-ras* targets/µL should contain approximately 10 amplifiable IgH targets/µL, e.g.
2. Round I PCR normally uses primers LJH and FR3A and program A. However, use LJH and FR2B and program B instead if the IgH gene lacks an FR3A binding site, or if one of the clone-specific primers is in CDR2.

Table 6
Use PCR to Test Whether Clone-Specific Primers Are Suitable for Limiting Dilution Quantification of Leukemia in Remission Marrows

Tubes	Template (2 µL)	Also add 2 µL of
1–2	50 ng diagnostic DNA	PCR-clean water
3–7	≈20 amplifiable *N-ras* targets/µL	Normal blood genomic DNA, 0.5 µg/µL
8–12	≈6 amplifiable *N-ras* targets/µL	Normal blood genomic DNA, 0.5 µg/µL
13–17	≈2 amplifiable *N-ras* targets/µL	Normal blood genomic DNA, 0.5 µg/µL
18–22	≈0.6 amplifiable *N-ras* targets/µL	Normal blood genomic DNA, 0.5 µg/µL
23–27	≈0.2 amplifiable *N-ras* targets/µL	Normal blood genomic DNA, 0.5 µg/µL
28–32	PCR-clean water	Normal blood genomic DNA, 0.5 µg/µL
33–37	PCR-clean water	PCR-clean water

3. Dilute 2.5 µL round I product in 250 µL PCR-clean water, for use as template in round II
4. Round II PCR uses clone-specific primers, hot start, and program E for leukemia-sized product < 150 bp, or program B for leukemia-size product >150 bp.
5. Analyze PCR products by electrophoresis. Expected results:
   a. Tubes 1 and 2: leukemia-size product, confirming amplification.
   b. Tubes 3–27: working from high to low DNA concentration: the proportion of tubes with leukemia-size product falls from 5/5 to 0/5; *and* the lowest DNA concentration to give leukemia-size product still gives *large* amounts of it. (For other results, *see* **Notes 17–20**.)
   a. Tubes 28–32: no leukemia-size product, confirming that the primers are specific.
   b) Tubes 33–37: no leukemia-size product, confirming lack of contamination.
6. Score the tubes as positive or negative for leukemia-size product, and use Poisson statistics to calculate how many amplifiable leukemia IgH targets are needed to generate the product (*see* **Subheadings 2.10.** and **2.11.**). Expected results, based on 132 patients: a median of 1.4 targets are needed. As explained before, the actual result is affected by several factors and usually ranges from 0.3–3 targets, but if >10 targets are needed *see* **Note 21**. Due to this variation, the best evidence for efficient amplification is that large amounts of product are obtained at the limit of dilution (*see* **Note 17**).
7. PCR conditions and primer combinations that give the expected results can be used to quantify leukemia by limiting dilution PCR, set out as follows.

3.6. Quantification of Leukemia by Limiting Dilution PCR

Confidence that the "clone-specific" PCR test will work well (*see* **Subheading 3.5.4.**) assists greatly in interpreting the results of these experiments.

Table 7
PCR to Estimate the Limit of Dilution for the Leukemia Marker Gene in a DNA Sample, and to Test for Inhibition

Tubes	Template (2 µL)	Also add 2 µL of:
1	DNA, 300 ng/µL	Dilute positive control DNA
2	DNA, 300 ng/µL	PCR-clean water
3	DNA, 100 ng/µL	Dilute positive control DNA
4	DNA, 100 ng/µL	PCR clean water . . .
. . . . .	etc.	. . .
25	DNA 0.3 pg/µL	Dilute positive control DNA
26	DNA 0.3 pg/µL	PCR-clean water
27,28	PCR-clean water	Dilute positive control DNA
29,30	PCR-clean water	PCR-clean water

3.6.1. Assessing the Proportion of PCR Targets That Can Be Amplified (see **Fig. 5***)*

1. Extract DNA from the test sample (*see* **Subheading 2.1.**).
2. Make a dilution series of the DNA (*see* **Subheading 2.10.**).
3. To calculate the fraction of PCR targets that can be amplified in the test sample, PCR is performed and interpreted as in **Subheading 3.5.3.** and **Table 5**. This information is used to calculate what proportion of nucleated cells belongs to the leukemia clone (*see* **Subheading 3.6.3., step 4**).

3.6.2. Initial Estimation of the Limit of Dilution

This experiment also confirms that amplification is efficient, particularly in high DNA concentrations, which often have to be studied to quantify low levels of leukemia, and are the concentrations most affected by inhibitors.

1. Prepare a dilute positive control from diagnostic DNA, with 10 amplifiable leukemia IgH targets/µL.
2. Set up and run a PCR as outlined in **Table 7**. Use conditions suitable for quantification as in **Subheading 3.5.4.**, with consensus primers in round I, and clone-specific primers in round II.
3. Analyze products by electrophoresis. Expected results:
   a. Tubes 27 and 28: leukemia-size product, confirming that the PCR worked efficiently.
   b. Tubes 29 and 30: no leukemia-size product, confirming no contamination.
   c. Other odd-numbered tubes: should all give leukemia-size product confirming efficient amplification of test DNA. (If some tubes do not, *see* **Note 22**.)

Table 8
Use PCR for Detailed Quantification of Leukemia in DNA Extracted from a Test Sample

Tubes	Template
1–5	Test sample, highest concentration
6–10	Test sample, next concentration (if there is one)
11 . . .	etc.
31 and 32	Dilute positive control DNA + 1 μg normal blood genomic DNA
33–37	PCR-clean water

   d. Other even-numbered tubes: leukemia-size product shows that the template added contained at least one leukemia IgH target.
4. Estimate the midpoint of the limit of dilution for the leukemia IgH target.

3.6.3. Detailed Quantification

1. Perform PCR with replicate samples at a limited number of DNA concentrations (*see* **Table 8**).
   Again, use conditions suitable for quantification as in **Subheading 3.5.4.**, with consensus primers in round I, and clone-specific primers in round II. If leukemia was detected in the test DNA, use up to six consecutive dilutions, five aliquots each, around the limit of dilution. Otherwise, use five aliquots of the most concentrated DNA sample.
2. Analyze samples by electrophoresis. Expected result:
   a. Tubes 31 and 32: leukemia-size product, confirming efficient amplification.
   b. Tubes 33–37: no leukemia-size product, confirming lack of contamination.
   c. Other tubes: depending on the level of leukemia, the highest concentration tested may show all, some, or no tubes with leukemia-size product; *and* working toward low DNA concentration, the proportion of tubes with leukemia-size product should fall to 0/5; *and* the lowest DNA concentration to give leukemia-size product still gives *large* amounts of product (for other results, *see* **Notes 17–20** and **23**).
3. If leukemia was detected: score the tubes as positive or negative for amplification of the leukemia-sized product, and use Poisson statistics to calculate the number of amplifiable leukemia IgH targets per 6 pg DNA (i.e., per marrow cell, assuming all PCR targets are amplifiable; *see* **Subheadings 2.10.** and **2.11.**). If leukemia was not detected: estimate an upper limit from the total amount of DNA studied, e.g., no leukemia in 600 pg DNA implies an upper limit of $<10^{-2}$.
4. To correct for DNA molecules that are not amplifiable, divide the result by the fraction of PCR targets that are amplifiable (*see* **Subheading 3.6.1., step 3**).
5. Expected result: leukemia may or may not be detected; its level can range from $>10^{-1}$ to $<10^{-6}$.

3.7. Clinical Significance and Interpretation of Levels of Leukemia in Marrow Aspirates

The marker gene is assumed to be stable, and to represent accurately all leukemia cells. These assumptions probably hold during induction treatment, although we have studied one patient who had at least two populations of leukemia cells, with different rearranged IgH genes, and different sensitivities to induction treatment. Study of more than one marker gene may provide more certainty. In the longer term, 25–30% of ALL cases undergo clonal evolution (25), and at relapse have altered or lost their IgH genes. Clone-specific PCR tests after induction treatment, which show little or no leukemia, should therefore be interpreted carefully. For long-term monitoring, alternative primer design strategies may be useful (e.g., ***26***).

More clinical research is needed to establish the significance of various levels of leukemia in marrow, because the prognosis can depend on when the test was performed, the patient's age, other clinical risk factors, and the drugs used. Nevertheless, the level of leukemia at the end of induction treatment is strongly correlated with outcome. In the patients we have studied, leukemia $>10^{-3}$ was associated with an overall 91% risk of relapse (21 of 23 patients). Patients with leukemia $< 2 \times 10^{-5}$ fared better, but a low level of leukemia in marrow at this stage does not rule out relapse, because it is a poor predictor of extramedullary relapse ***(3,4,16)***.

4. Notes

1. This method can be used to estimate the fraction of PCR targets that are amplifiable in any human DNA sample, provided that the PCR targets are similar in size to the *N-ras* target, and *N-ras* remains a single-copy gene.
2. FR2B-ELJH also amplifies a nonspecific product of 225 bp, which is smaller than IgH products, and, unlike them, is always the same size in every patient.
3. To obtain DNA samples from each rearranged IgH gene, repeat **Subheading 3.1.** with 6, 20, 60, or 200 pg DNA/PCR tube, 10 tubes each. In this limiting dilution experiment, one DNA concentration should yield leukemia IgH product in about half its tubes, implying an average of 1 IgH target per tube. Alternatively, the IgH products can be cloned ***(1,14)***.
4. The leukemia cells lack an IgH gene that can be amplified by the primers. The smear is from IgH genes from normal lymphocytes.
5. Same explanation as **Note 4**, and presence of a few bands, rather than a smear, suggests a low concentration of amplifiable IgH targets ***(27)***.
6. Large-volume PCRs may work poorly. Perform several 100 µL PCRs, and combine the products.
7. If the (+) and (–) strand sequences differ by a few bases: most differences can be resolved by careful study and comparison of the two chromatograms. Areas

poorly sequenced on one strand are usually sequenced clearly on the other. Otherwise, sequence the (–) strand of DNA sample 1 and the (+) strand of DNA sample 2, and compare all four chromatograms (two DNAs, both strands each).

8. If sequence information close to the sequencing primer is lost: check the chromatogram from the complementary DNA strand, where the sequence should be at the opposite end, and clear. Otherwise, ask your sequencer service to reanalyze the data with the start point closer to the bottom of the gel image.
9. Sequence that is unreadable, due to a low signal strength, or more than two strong peaks at many chromatogram positions, implies low concentration DNA, chemically impure DNA, or DNA containing several sequences. Consult the manufacturer's literature or your sequencing service before attempting to sequence other samples.
10. If the (+) and (–) strand sequences are different IgH rearrangements: the leukemia cells lack an IgH gene that can be amplified by the primers. IgH genes from normal lymphocytes have been amplified instead (*see* **Notes 4** and **5**).
11. If exactly two signals are present at most chromatogram positions: the DNA may have contained two IgH rearrangements that were very similar in size (*see* **Note 3**).
12. Many germline D and J segment sequences are tabulated in **ref. *24***. Type several germline D (or J) segment sequences one after the other in a single file. Some software can display homology as plot with the D (or J) segment sequences along the *x*-axis, the rearranged IgH sequence on the *y*-axis, and a diagonal line showing homology between them. The length of this line allows homology to each D (or J) segment to be rapidly compared.
13. The FR3A primer binds to a highly conserved site ending in . . . TATTACTGT, near the end of V (*see* **Fig. 3**). During recombination, DNA may be removed sequentially from the end of V, including part or all of the consensus GCG (A/G)G(A/G)GA and sometimes part or all of the FR3A binding site. If N, D, and J segments are present, clone-specific primers can still be designed.
14. DNA can also be removed from the end of J segments during recombination, leaving no homology beyond the consensus J primer's binding site.
15. If the PCR test is not specific for the leukemia IgH sequence: repeat, trying the following variations: new clone-specific primers whose 3′ ends differ by a few bases from those initially designed; 20 ng of primer per PCR; PCR program A or B.
16. Primers almost always amplify the DNA sample that was sequenced, unless their sequences do not match, or a synthesis failure produced very little full-length primer. To test individual primers, repeat **Subheading 3.5.2.**, with each clone-specific primer paired with a consensus primer.
17. PCR yields faint bands at the limit of dilution: the key issue is that all tubes must be scored accurately for the presence of PCR targets in the original template of genomic DNA. PCR efficiency can vary from tube to tube. If amplification is weak, some tubes might have received a PCR target but yielded too little PCR product to be visible, and were mistakenly scored as negative. Repeat, with new PCR mix.

18. If some or all tubes at the highest DNA concentration are negative, or some or all at the lowest DNA concentration are positive, the concentrations chosen may not span the limit of dilution. Test higher or lower DNA concentrations, and combine results with the current experiment, for statistical analysis.
19. If the data do not fit a Poisson distribution ($\chi^{2;}$ $p < 0.05$): usually due to chance. Repeat the experiment, pool the data, and reanalyze. If the data still do not fit, there may be an error in preparing the dilution series.
20. If, working from high to low DNA concentration, the proportion of tubes positive for amplification remains at 1/5 or 2/5 *for several concentrations*: possibly there is a low level of contamination, or a clone-specific PCR may amplify other IgH genes. (To improve specificity, *see* **Note 15.**)
21. Assuming efficient amplification, the marker sequence may belong to a subpopulation of cells in the diagnostic sample. Quantification can proceed, assuming that the subpopulation represents all leukemia cells.
22. If some DNA concentrations, usually the higher concentrations, show no leukemia-size product: occasionally DNA samples contain PCR inhibitors, e.g., heparin, phenol. For quantification, either remove the inhibitor, or study low DNA concentrations where the inhibitor is too dilute to affect PCR.
23. If clone-specific PCRs that appeared suitable for limiting dilution quantification lack specificity when used to study patient DNA: tests with normal peripheral blood may not accurately model the background DNA in the patient. (To improve specificity, *see* **Note 15**.)

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8

NPM-ALK Reverse Transcriptase–Polymerase Chain Reaction for Detecting the t(2;5) of Non-Hodgkin's Lymphoma

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1. Introduction

The development of modern molecular biology techniques and their use in characterizing the genetic abnormalities that are of pathogenic significance in non-Hodgkin's lymphoma (NHL) now provides a means to diagnose and rationally subcategorize these neoplasms in addition to the more traditional use of morphologic and immunophenotypic criteria. The shortcomings of traditional methods of NHL diagnosis and classification have been especially evident within the morphological subset commonly referred to as the large-cell lymphomas, which comprise approx 25% and 40% of NHL in children and adults, respectively ***(1)***. The marked cytological, immunological, and clinical heterogeneity of this group of tumors suggests that it is comprised of several biologically different neoplasms, but morphological and immunophenotypic subclassification schema have failed to identify meaningful subsets within the large-cell lymphomas. Recently, however, two recurrent genetic abnormalities in large-cell NHL—activation of the *BCL6/LAZ3* zinc finger gene located at 3q27 (altered in approx 30% of large-cell lymphomas ***[2–4]***) and the *NPM-ALK* fusion gene ***(5–7)*** produced by the t(2;5)—have been analyzed to now permit the identification of patient subsets that have reproducibly different therapeutic responses and survival rates in most studies, both of these molecular genetic subtypes appearing to have a superior prognosis compared to those cases lacking these gene abnormalities.

The t(2;5)(p23;q35) was originally identified as a recurrent cytogenetic abnormality in cases of anaplastic large-cell lymphoma (ALCL) that express

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the Ki-1 (CD30) antigen, a cytokine receptor for a ligand related to the tumor necrosis factor family ***(8)***. Because of their anaplastic features, these tumors, which are primarily of T-cell lineage (75%), are frequently misdiagnosed as other conditions including Hodgkin's disease, malignant histiocytosis, mycosis fungoides, poorly differentiated carcinoma metastatic to lymph nodes, or even viral infections. Clinically, Ki-l+ lymphomas/ALCL are aggressive tumors that produce frequent extranodal disease involving skin, bone, soft tissue, gastrointestinal tract, and lung and that exhibit a bimodal age distribution similar to that observed in Hodgkin's disease ***(9)***.

Despite the historical association of the t(2;5) with Ki-1+ lymphomas/ALCL, molecular studies performed using the reverse transcriptase-polymerase chain reaction (RT-PCR) method described here, as well as other techniques including anti-ALK immunostaining and t(2;5) DNA-PCR, indicate that the translocation can also occur in occasional large-cell lymphomas with immunoblastic or diffuse morphology ***(6)***. Indeed, recent studies have clearly shown that lymphomas that express a truncated ALK protein (so-called "ALK lymphomas" or "ALKomas") exhibit a broad morphologic spectrum and that morphology alone cannot distinguish these tumors ***(10,11)***. Moreover, only slightly more than half of the large-cell lymphomas with anaplastic morphology and slightly less than two-thirds of the Ki-l+ cases analyzed in molecular diagnostic studies contain the t(2;5). Thus, although clearly most frequent in Ki-1+ lymphoma/ALCL, this molecular genetic subtype of NHL can occur in all morphologic types of large-cell lymphoma and is independent of Ki-l antigen status.

Overall, based on data from those studies performed to date, approximately 40% of ALCL (or about 5% of all NHL) contains the t(2;5) (*see* **Table 1**). Although still not unequivocally determined, most reports correlating overall (OS) and failure-free (FFS) survival with the presence or absence of the t(2;5) in ALCL have shown those cases containing the rearrangement to have a clearly superior prognosis (e.g., 5-yr OS and FFS rates of approx 80% vs approx 40% for translocation-positive or negative cases, respectively) ***(6,7)***. It is important to note that although a few small studies have suggested that the t(2;5) can be found in Hodgkin's disease, the bulk of evidence suggests the translocation is rarely, if ever, present in either Hodgkin's disease or in secondary ALCL (ALCL that occurs due to histological transformation of other NHL subtypes) ***(6,7,12)***. It currently remains unclear, however, whether CD30+ primary cutaneous lymphoma and its benign counterpart, lymphomatoid papulosis, contain the t(2;5) in some cases ***(13–15)***.

The cloning of the t(2;5) demonstrated the rearrangement to fuse the chromosome 5 gene locus encoding nucleophosmin (NPM), a nucleolar phos-

Table 1
Frequency of the t(2;5) in ALCL

Reference	Method	Number of patients	% Positive
Bullrich et al., *Cancer Res.* **54:**2873–2877, 1994.	Southern blot	16	13
Downing et al., *Blood* **85:**3416–3422, 1995.	RT-PCR	28	54[a]
Elmberger et al., *Blood* **86:**3517–3521, 1995.	RT-PCR	22	36
Herbst et al., *Blood* **86:**1694–1700, 1995.	ISH, IM	85	19
Hutchinson et al., *Annals of Oncol.* **8(Suppl 1):**37–42, 1997.	IM	48	50
Lamant et al., *Blood* **87:**284–291, 1996.	C, IM, RT-PCR	56	73
Lopategui et al., *Am. J. Path.* **146:**323–328, 1995.	RT-PCR	37	16
Nakamura et al., *Am. J. Surg. Pathol.* **21:**1420–1432, 1997.	IM	67	64
Ngan, *Modern Pathology* **8:**118a, 1995.	RT-PCR	15	13
Orscheschek et al., *Lancet* **345:**87–90, 1995.	RT-PCR	5	80
Pittaluga et al., *Am. J. Path.* **151:**343–351, 1997.	IM	61	21
Pulford et al., *Blood* **89:**1394–1404, 1997.	IM	77	55
Sarris et al., *Leukemia Lymphoma* **29:**507–514, 1998.	DNA-PCR	38	16
Shiota et al., *Blood* **84:**3648–3652, 1994; *Blood* **86:**1954–1960, 1995.	IM, RT-PCR	105	29
Waggot et al., *Br. J. Haematol.* **89:**905–907, 1995.	DNA-PCR	5	100[b]
Weisenburger et al., *Blood* **87:**3860–3868, 1996.	C	10	50
Wellman et al., *Blood* **86:**2321–2328, 1995.	RT-PCR	24	46
Yee et al., *Blood* **87:**1081–1088, 1996.	RT-PCR, IM	49	12
SUM		743	38

[a]Only cases that were morphologically ALCL.

[b]Excludes these five cases because this was not a prevalence study.

Abbreviations: C, cytogenetic; RT, reverse transcriptase; ISH, *in situ* hybridization; IM, immunocytochemistry.

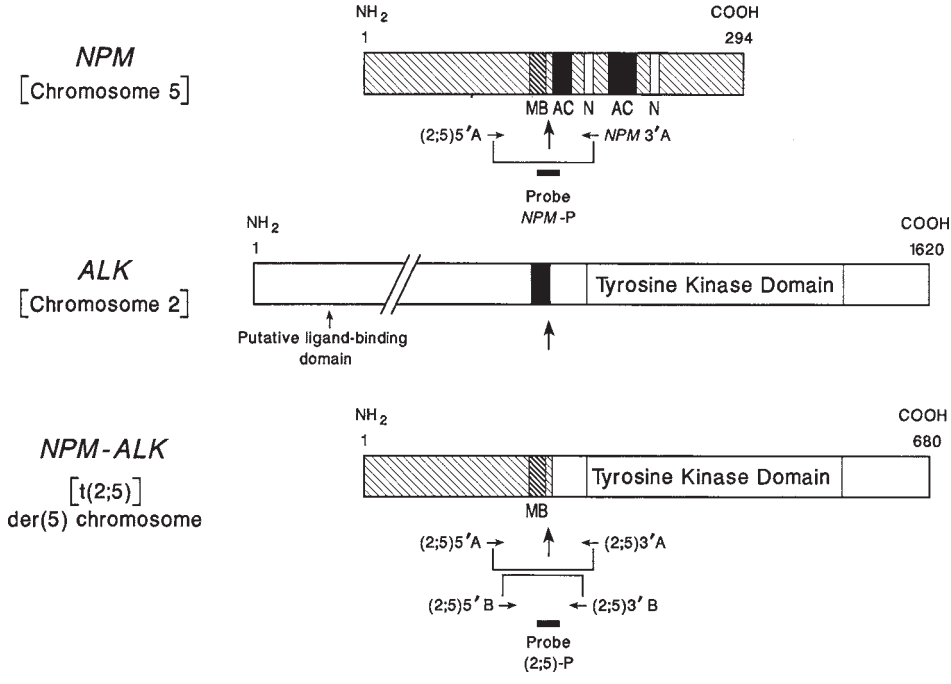

Fig. 1. Schematic representation of the proteins encoded by the normal *NPM* and *ALK* genes and by the *NPM-ALK* fusion gene present on the derivative 5 chromosome produced by the t(2;5). NPM is an ubiquitously expressed nucleolar phosphoprotein that normally functions as a shuttle protein for ribonucleoproteins from the nucleolus to the ribosomes in the cytoplasm. The metal binding (MB), acidic amino acid clusters (AC) and nuclear localization signals (N) of NPM are indicated. ALK is a receptor tyrosine kinase of the insulin receptor subfamily that is normally expressed only in neural tissues. The presumed ligand-binding, transmembrane (TM), and intracytoplasmic kinase catalytic domains of ALK are shown. The positions at which the fusion junctions occur in the t(2;5) translocation are indicated by arrows. The *NPM-ALK* chimeric gene produces an 80-kDa product consisting of the amino-terminal portion of NPM fused to the cytoplasmic domain of ALK. The approximate position of the oligonucleotide primers and probes used for RT-PCR analysis of the t(2;5) are indicated.

phoprotein to the chromosome 2 encoding anaplastic lymphoma kinase (ALK) ***(5)***. The chimeric *NPM-ALK* fusion gene and transcript is encoded on the der(5) chromosome and produces a fusion protein consisting of the amino-terminal segment of NPM linked in-frame to the kinase domain of ALK (*see* **Fig. 1**). NPM is a highly conserved (and ubiquitously expressed) 38-kDa nonribosomal RNA-binding protein that shuttles ribosomal ribonucleoproteins

between the nucleolus and the cytoplasm and is involved in the late stages of preribosomal particle assembly ***(16,17)***. ALK is a member of the insulin receptor subfamily, having greatest homology to leukocyte tyrosine kinase (LTK)***(5)***. The ligand(s) that bind ALK and the normal functions of this receptor tyrosine kinase remain unknown; however, it is clear that the normal ALK receptor is limited in its expression to the central and peripheral nervous systems ***(18,19)***. As a result of the t(2;5), transcription of the portion of *ALK* encoding its kinase domain is driven by the strong *NPM* gene promoter, leading to its inappropriate expression in lymphoid cells (in which *ALK* is usually transcriptionally silent). Furthermore, as a result of its fusion with NPM, the ALK kinase domain is constitutively activated and is relocalized from its normal position in neural cells at the inner surface of the cell membrane to within both the cytoplasm and nucleus of lymphoma cells ***(20,21)***. The oncogenic ability of the chimeric NPM-ALK protein can be readily demonstrated in vitro using immortalized rodent fibroblast lines such as NIH-3T3 and in vivo in mice ***(21–24)***.

The molecular characterization of the t(2;5) has permitted the development of an RT-PCR assay using primers derived from *NPM* and *ALK* sequences bracketing the *NPM-ALK* fusion junction that allows rapid and sensitive detection of the translocation in clinical material. We and others have used this assay to successfully detect *NPM-ALK* transcripts in several hundred cases of NHL (*see* **Table 1**). Because variant *NPM-ALK* fusion junctions occur only *very* rarely ***(6,25)***, the RT-PCR assay described in this chapter should permit detection of the t(2;5) in essentially all cases containing the translocation.

It is worth noting here that several recent studies have demonstrated that PCR using genomic DNA extracted from tumors is also a highly efficient and reliable technique for detection of the t(2;5) ***(15,26,27)***. Genomic DNA-PCR is feasible because the *NPM* and *ALK* introns in which the breakpoints almost invariably occur are small (approximately 1 and 2 kilobases [kb], respectively) ***(28)***. Two theoretical advantages of *NPM-ALK* DNA-PCR compared to RT-PCR are (1) the greater stability and ease of manipulation of DNA versus RNA, and (2) the fact that differently sized DNA-PCR amplicons are generated from individual tumor samples (unlike RT-PCR in which the amplicon sizes are identical), meaning that the ever-present problem of sample cross-contamination in the laboratory is not as serious a concern. The *NPM-ALK* RT-PCR primers described here are also suitable for DNA-PCR; readers interested in the DNA-PCR method are referred to the citations noted previously for details.

It is also important to point out that although the vast majority of lymphomas in which an abnormal ALK protein is expressed contain the typical *NPM-ALK* fusion, approx 15% of ALK-positive lymphomas contain variant

rearrangements that result in the fusion of a truncated *ALK* gene to partners other than *NPM*, such as *TPM3* (tropomyosin-3)–*ALK*, *TFG* (*TRK*-fused gene)–*ALK*, *ATIC* (5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase)–*ALK*, or CLTCL (clathrin chain polypeptide-like)–*ALK* ***(29–35)***. None of these abnormalities, which include the inv(2)(p23q35) (that generates ATIC-ALK) and the t(1;2)(q25;p23) (that produces TPM3-ALK) ***(20,30,36,37)***, are detected by NPM-ALK PCR assays, of course. Two methods are available currently that permit detection of theoretically all ALK fusion-positive NHLs: (1) FISH using *ALK* probes that either span or blank the gene locus, and (2) immunostaining using anti-*ALK* antibodies specific for the carboxy-terminal portion of the protein ***(10,11,36)***. In most clinical diagnostic laboratories, anti-ALK immunostaining is used. Caveats to the use of ALK immunostaining do exist, however, including the existence of a rare large B-cell lymphoma that occurs mainly in adults, is associated with a poor prognosis, and expresses the full-length ALK receptor (not a truncated ALK) ***(38)***. In addition, it appears that the majority of neuroblastomas express full-length ALK protein, although the pathogenic importance of ALK in these tumors is unclear at this time ***(39)***. Finally, it has very recently been noted that a subset of inflammatory myofibroblastic tumors (IMTs), uncommon spindle cell proliferations having a distinctive pseudosarcomatous inflammatory appearance that typically occur in the soft tissue and viscera of children and young adults, express ALK fusion proteins such as TPM3-ALK, TPM4 (tropomyosin-4) - ALK, CLTCL-ALK, and RanBP2 (Ran-binding protein 2)–ALK ***(40,41)***. These constitutively-activated ALK fusions in IMTs join amino-terminal portions of TPM3, TPM4, CLTCL, or RanBP2 that contain dimerization motifs to the same segment of ALK as found in NPM-ALK. Although full-length ALK-positive B-cell lymphomas, neuroblastomas, and IMTs stain positive with ALK antibodies, the clinical, morphologic, and immunophenotypic features of these tumors are unique compared to NHLs that express ALK fusion proteins, thus permitting their recognition.

2. Solutions and Materials

2.1. RNA Extraction

There are a number of methods that can be used for RNA extraction. We are currently using Purescript RNA isolation kits from Gentra Systems (Research Triangle Park, NC). If appropriate kits are unavailable, the procedure outlined here works well. It uses a lysing solution containing guanidinium thiocyanate to inactivate RNases, followed by a number of extractions ***(42)***. This method gives a reasonable yield (10–20 μg) of RNA that is relatively free from contaminating DNA from 1×10^7 starting cells. Any other method of extraction

that gives comparable quality RNA can be used. Most commercially available extraction kits have the advantage of eliminating the use of organic solutions like phenol/chloroform and are much more rapid than traditional RNA preparation methods.

1. Diethylpyrocarbonate (DEPC)-treated water: Add 200 µL of DEPC (Sigma, St. Louis, MO) to 1 L of water. Shake well, then loosen cap and incubate for 2–8 h at 37°C. Autoclave, then shake again to remove DEPC breakdown products.
2. 10% Sarcosine: Add 250 mL of DEPC-treated water to 25 g of *N*-lauroyl sarcosine (Sigma), filter through a 0.45-µM sterile filter system, and store at room temperature.
3. 0.75 *M* Citric acid: Add 400 mL of DEPC-treated H_2O to 78.75 g of citric acid (Sigma), then bring volume up to 500 mL.
4. 0.75 *M* Sodium citrate, pH 7.0: Add 400 mL of DEPC-treated H_2O to 110 g of sodium citrate (Sigma), pH to 7.0 with 0.75 *M* citric acid, then make up to 500 mL.
5. Denaturing solution: 4 *M* Guanidinium thiocyanate, 25 m*M* sodium citrate, pH 7.0; 0.5% sarcosine. Add 117.2 mL of DEPC-treated water to a 100-g bottle of guanidinium thiocyanate (Fluka Chemicals, Ronkonkoma, NY), then add 10.5 mL of 10% sarcosine, 7.04 mL of 0.75 *M* sodium citrate, pH 7.0, swirl container to mix, and heat at 65°C to aid in dissolution if necessary.
6. Solution D: Add 360 µL of β-mercaptoethanol (Sigma) to 50 mL of denaturing solution. This solution should be aliquoted and can be stored for 1 mo at room temperature.
7. Phenol (water-saturated), pH 6.6 (Amresco, Solon, OH). Store at 4°C.
8. Chloroform (Fisher, Pittsburgh, PA): Store at room temperature in a dark bottle.
9. Isoamyl alcohol (Sigma): Store at room temperature.
10. Chloroform/isoamyl alcohol: Add 4 mL of isoamyl alcohol to 96 mL of chloroform. Store at room temperature in a dark bottle.
11. Isopropanol (Fisher): Store at room temperature.
12. 75% Ethanol: Add 25 mL of DEPC-treated water to 75 mL of absolute ethanol (Aaper, Shelbyville, KY).
13. Glacial acetic acid (Fisher): Store at room temperature.
14. 2 *M* Sodium acetate (NaOAc, Fisher), pH 4.0. Add 15 mL of DEPC-treated water to 136 g of sodium acetate; add glacial acetic acid until pH is 4.0, then make up to 500 mL. Store at room temperature.
15. RNasin (Promega, Madison, WI): 40 U/mL. Store at 20°C.
16. Labconco RNA isolation hood (optional): Model 5102 (Labconco, Kansas City, MO).
17. Pipettors: P1000, P200, P20 (Rainin, Woburn, MA).
18. 1.7 mL siliconized microfuge tubes (PGC Scientific, Frederick, MD).
19. SpeedVac (Savant, Farmingdale, NY).
20. Water bath (Fisher), set at 37°C.
21. Eppendorf microfuge (Brinkman Instruments, Westbury, NY).

2.2. RT-PCR of the t(2;5)

1. Oligonucleotides (required for primer extension):
   a. (2;5)5′A, 5′-GCTTTGAAATAACACCACCAG-3′, 5′ oligo for t(2;5).
   b. (2;5)3′A, 5′-TAGTTGGGGTTGTAGTCGGT-3′,3′ oligo for t(2;5).
   c. (2;5)5′B, 5′-CCAGTGGTCTTAAGGTTG-3′, 5′ nesting oligo for t(2;5).
   d. (2;5)3′B, 5′-TACTCAGGGCTCTGCAGC-3′, 3′ nesting oligo for t(2;5).
   e. (2;5)5′A, same as above, 5′ oligo for *NPM* control.
   f. *NPM3′A*, 5′-CAGACCGCTTTCCAGATATAC-3′, 3′ oligo for *NPM* control.
   g. (2;5)-P, 5′-AGCACTTAGTAGTGTACCGCCGGA-3′, probe for t(2;5).
   h. *NPM*-P, 5′-GTGCTGTCCACTAATATGCAC-3′, probe for *NPM* control.

   Primers (2;5)5′A and (2;5)3′A are used to amplify the t(2;5)-derived *NPM-ALK* fusion gene transcripts and generate a 177-bp product. Primers (2;5)5′A and *NPM3′A* are used as a positive control to detect *NPM* gene transcripts, which are ubiquitously expressed at high levels, in order to check the quality of the RNA. This primer pair generates a 185-bp *NPM* PCR product. Primers (2;5)5′ B and (2;5)3′ B are nesting oligos that can be used to further amplify the t(2;5) PCR fusion product for increased sensitivity of detection, if necessary. This primer pair generates a 123-bp *NPM-ALK* product. Probe (2;5)-P is a junction-specific probe to detect the *NPM-ALK* fusion product. Probe *NPM-P* is used to detect the control *NPM* PCR product. All primers and probes should be aliquoted and stored at –80°C.
2. DMSO (dimethyl sulfoxide, Sigma) (*see* **Note l**): Stored at room temperature.
3. dNTPs (deoxynucleotide triphosphates, Perkin-Elmer Cetus, Norwalk, CT). A stock dNTP solution is made that is 1.25 m*M* with respect to each dNTP for both cDNA production and PCR amplification. Store at –70°C.
4. DTT (dithiothreitol, Gibco-BRL, Grand Island, NY) (*see* **Note 2**): the stock solution is 0.1 *M* and is stored at –70°C.
5. DEPC-treated H_2O. (*See* **Subheading 2.1.1.**)
6. PCR buffer: 10X by Perkin-Elmer Cetus: 100 m*M* Tris-HCl, pH 8.3, 500 m*M* KCl, 15 m*M* $MgC1_2$, 0.01% gelatin; 5X buffer by Invitrogen (San Diego, CA); 300 m*M* Tris-HCl, pH 9.0; 75 m*M* $(NH_4)_2SO_4$, 10 m*M* $MgCl_2$. Both buffers are stored at –70°C.
7. Reverse transcriptase (RT): Stock is Moloney Murine Leukemia Virus RT (Life Technologies, Grand Island, NY) at a concentration of 200 U/µL. Store at –20°C.
8. RNasin (*see* **Note 3**): stock is 40 U/µL (Promega). Store at –20°C.
9. *Taq* DNA polymerase: Stock is 5 U/µL (Perkin-Elmer Cetus). Store at –20°C.
10. Thermocycler (Perkin-Elmer Cetus 9600 or 480).
11. Eppendorf microcentrifuge.
12. 37°C Water bath.
13. 95°C Heating block (Equatherm, Melrose Park, IL).
14. Sterile hood (optional but desirable) (Baker, Sanford, ME).
15. Gloves.

16. Microfuge tubes-siliconized.
17. Aerosol resistant barrier pipet tips (ART tips, Molecular Bioproducts, San Diego, CA).
18. Pipettors.
19. Ice trays.

2.3. Gel Electrophoresis and Southern Analysis

1. l0X Tris-borate electrophoresis buffer (TBE): 108 g Tris base, 55 g boric acid, and 40 mL of 0.5 *M* ethylenediaminetetraacetic acid (EDTA). Made up to 1 L and autoclaved. Store at room temperature.
2. 6X Loading buffer: 0.25% Xylene cyanol, 0.25% bromophenol blue, and 30% glycerol.
3. Ethidium bromide: Stock 10 mg/mL, final concentration 10 µg/mL in 1X TBE.
4. Agarose.
5. Nylon or nitrocellulose hybridization membranes.
6. 20X Standard saline citrate (SSC) stock solution: 3 *M* Sodium chloride, 1 *M* sodium citrate.
7. 20% Sodium dodecyl sulfate (SDS) stock solution: 20 g of SDS in 100 mL of water. Wear a mask when making this solution and gently heat to dissolve, but do not boil.
8. (2;5)-P oligonucleotide (1 µ*M* stock).
9. Paper towels.
10. l0X T4 end-labeling buffer (manufacturer's specification).
11. T4 polynucleotide kinase (10 U/reaction).
12. γ^{32}P dATP (specific activity >3000 Ci/mmol).
13. Distilled water.
14. X-ray film.

3. Methods

3.1. RNA Extraction

3.1.1. General Considerations

Because RNA is easily degraded, extreme care should be taken when handling samples (*see* **Notes 4** and **5**). Ideally, RNA should be extracted in an area dedicated solely for that purpose and away from areas used for cloning and DNA work (*see* **Note 6**). Gloves should be worn at all times, and work areas and instruments should be decontaminated with bleach or ultraviolet (UV) irradiation. All solutions, with the exception of Tris, should be treated with the RNase inhibitor DEPC. Solutions are treated for 2–8 h with 0.1% DEPC at 37°C, followed by autoclaving as described in **Subheading 2.1.1.** Tris solutions should be made with DEPC-autoclaved water. Sterile disposable plasticware should be used whenever possible; all glassware used should be DEPC-treated.

Stock reagents required for RNA extraction should be prepared in volumes small enough to use completely within a relatively short period (l–2 mo), as reagents such as phenol, "solution D," and chloroform have a limited shelf life. It is also desirable to limit the number of times one enters the same reagent bottle to decrease the possibility of sample cross contamination. Because of the extreme sensitivity of RT-PCR (allowing the detection of one abnormal cell in 100,000 cells in the case of the assay described here), it is necessary to take every precaution to reduce the risk of cross contamination of samples. With this goal in mind, oligos and reagents used for either the reverse transcription (RT) reaction or the PCR should be aliquoted to single-run volumes so that a vial is thawed, used, and then discarded. Separate sets of pipettors should be dedicated for RNA extraction, RT-PCR amplification, and postamplification analysis. The lab should have dedicated areas or, ideally, separate rooms, designated for RNA extraction, RT-PCR amplification, and postamplification analysis. Large RNA extractions from positive or negative control samples should be done in a separate area away from patient RNA extractions. Under no circumstances should PCR product be brought back into the area where RT-PCR is set up.

3.1.2. Specific Procedures

1. Aliquot 400 µL of solution D into 1.7-mL microfuge tubes, add 2 µg of glycogen, and label tubes with pertinent information (patient name, ID number, date, and so on). In addition to the patient samples, one tube is needed for a negative extraction control (no cells added to this tube), one tube for a negative cell line control (e.g., HL60) ***(43)***, and one tube for a positive cell line control (a t[2;5]-positive lymphoma cell line such as SU-DHL-l) ***(44)***.
2. To each tube add $1–2 \times 10^7$cells.
3. Quick spin for 1 s at 2000*g* in a microcentrifuge.
4. Add 40 µL of 2 *M* NaOAc to each sample. Close lid and flick to mix.
5. Add 400 µL of water-saturated phenol to each tube. Close lid and flick to mix.
6. Add 82 µL chloroform/isoamyl alcohol (24 : 1) to each tube.
7. Vortex each tube for l0 s, then place on ice for 15 min.
8. Centrifuge all samples at 16,000*g* for 20 min at 4°C.
9. Transfer the top aqueous phase to a new 1.7-mL microfuge tube and place on ice. (Be careful not to remove any of the organic phase or white interface.)
10. Add an equal volume of isopropanol to the aqueous layer of each sample.
11. Vortex each sample for 5 s, and place on dry ice for 30 min (or at –70°C overnight).
12. Centrifuge samples at 16,000*g* for 15 min at 4°C.
13. Remove the supernatant from each sample, taking care not to disturb the small white RNA pellet on the bottom and side wall of the tube.
14. SpeedVac each sample until dry.

15. Resuspend pellets in 300 µL of solution D, making sure pellet is completely dissolved.
16. Add 300 µL of isopropanol to each sample.
17. Vortex each sample for 5 s, then quick spin by microfuging at 2000*g* for l–2 s.
18. Place each sample on dry ice for 30 min (or overnight at –70°C).
19. Centrifuge each sample at 16,000*g* for 10 min at 4°C.
20. Remove the supernatant from each sample, taking care not to disturb the small white RNA pellet on the bottom and side wall.
21. Add 500 µL of chilled 75% ethanol to each sample.
22. Vortex each sample until pellet is dislodged from the bottom of the microfuge tube.
23. Centrifuge each sample at 16,000*g* for 10 min at 4°C.
24. Carefully remove the supernatant from each sample without disturbing the RNA pellet.
25. SpeedVac each sample for 10 min (pellets should appear dry).
26. Resuspend pellets in 10 µL DEPC-water and add 1 µL RNasin.
27. Vortex each sample several times to dissolve RNA, then quick spin.
28. Store samples frozen at –70°C.

3.2. RT-PCR

3.2.1. Controls

Each RT-PCR will typically include the reverse transcription and amplification of RNA extractions from several patients and controls. As mentioned in the RNA extraction procedure, several negative and positive controls are used, including the following:

1. A negative RNA control. This is a tube that had no cells added prior to the extraction that is used to ensure that all of the RNA extraction solutions are free of contaminating nucleic acids.
2. An HL60 (or comparable cell line) negative RNA control. A cell line, such as HL60, which does not contain the t(2;5) translocation, should also be included as a negative control ***(43)***. RT-PCR of RNA from such a cell line should be positive for the control product (from the normal *NPM* gene) but negative for the t(2;5) *NPM-ALK* fusion product. If this sample is positive for the t(2;5), it indicates that contamination occurred during the run and makes all other results uninterpretable.
3. A positive SU-DHL-1 RNA control. The SU-DHL-1 lymphoma cell line contains the t(2;5) ***(44)***. Therefore, RT-PCR of RNA from this cell line should be positive for both the *NPM* control and the t(2;5)-derived *NPM-ALK* product. If no *NPM-ALK* signal is present, it indicates that a reagent used, often an oligo, was not good and renders all other results uninterpretable. Again, due to the extreme sensitivity of RT-PCR, a number of precautions should be taken to eliminate possible sources of contamination. All stocks of reagents, with the possible exception of enzymes, should be in single-run aliquots. The RT-PCR must be set

up in a hood, in a separate room or area, which can be sterilized with UV light between runs. Disposable isolation gowns and gloves should be worn during RT-PCR, and all reagents needed should be brought on wet or dry ice so that once the RT-PCR run is started it is not necessary to leave the hood area until the samples are ready to go into the thermocycler. If it becomes necessary to leave, a new pair of gloves and gown should be put on at reentry.

3.2.2. RT Reaction

Each RT run should include separate tubes for each patient sample, a "No RNA" negative control, a negative control cell line RNA, and a positive cell line RNA. RT is done in a 20-µL total volume. If possible, all of the following steps should be done in a sterile hood.

1. Label two 1.7-mL centrifuge tubes, one for the t(2;5) and one for the *NPM* control.
2. Add the following amounts of each reagent to the tubes for **each sample and control to be analyzed** as shown in **Table 2**.
3. Label 0.5-mL microfuge tubes, two for each patient and controls. Aliquot patient and control RNAs (0.5–1 µL of total RNA from each sample) into appropriate tubes and make up to 11 µL. Heat at 95°C for 5 min. (Remember to set up for two PCR runs: one for the t(2;5) and one for the *NPM* control.)
4. Quick-spin samples in a microcentrifuge, then set on ice.
5. Add 9 µL of either the t(2;5) or *NPM* RT mix to each RNA tube (*see* **Note 7**).
6. Incubate in a 37°C water bath for 1 h.

3.2.3 PCR Reaction

While the RNA is being reverse transcribed, the PCR oligo mixes can be set up. Alternatively, the PCR oligo mixes can be made at the same time as the RT mixes with the exception of the *Taq* polymerase enzyme, which should be added just prior to use. PCR mixes are made up in a total volume of 80 µL/reaction; this volume is added to each 20 µL vol of cDNA following RT.

1. Make up the following (*see* **Table 3**):
2. If the *NPM-ALK* fusion product is barely detectable or absent, a nesting PCR reaction can be run using 5% of the reaction mix from the first run. This starting volume is brought up to 20 µL, then 80 µL of the nesting oligo mix is added. (*see* **Table 4**).
3. Samples are overlaid with one drop of mineral oil unless the run is done in a Perkin-Elmer Cetus 9600 PCR machine, in which case oil is not needed. To obtain higher specificity, the PCR tubes are run on a touchdown program, in which the annealing temperature starts out high, then drops 1°C each cycle until it reaches the ideal annealing temperature at which the majority of the cycles are then performed (*see* **Table 5**):

Table 2
Composition of RT Reaction Mixes

RT mixes		Final concentration (in 20 µL)
10X PCR buffer (Perkin-Elmer Cetus)	2 µL/reaction	1X
dNTPs (1.25 m*M* stock)	2 µL/reaction	0.125 m*M*
DTT (0. 1 *M* stock)	2 µL/reaction	10 m*M*
oligo (2;5)3′A for t(2;5) mix *or* oligo *NPM* 3′A for *NPM* control (use 10 pmol of the appropriate oligo per run)		0.5 pmol/µL
RNasin (40 U/µL)	0.5 µL/reaction	1 U/µL
Reverse transcriptase (200 U/µL)	1 µL/reaction	10 U/µL
DEPC-treated water	Make up to 9 µL/reaction	

3.3. Gel Electrophoresis and Southern Analysis

Following the completion of PCR:

1. Remove 20 µL from each reaction and transfer to a new tube (if oil was used, be sure to wipe off the tip).
2. Add loading dye.
3. Run each sample on a 1.2% agarose TBE gel at 80 V with appropriate size markers.
4. Southern transfer the gel onto a nitrocellulose or nylon membrane (*see* **Note 8**).
5. Hybridize with the *NPM-ALK* junction-specific probe, (2;5)-P, which has been end-labeled with ^{32}P using polynucleotide kinase.
6. Wash the blots at room temperature for 30 min in 2X SSC, 0.1% SDS, then for 30 min at 50°C, also in 2X SSC, 0.1% SDS.
7. Autoradiograph and develop after 4 h exposure (*see* **Notes 9–13**).

4. Notes

1. DMSO reduces secondary structure of the template that can interfere with extension by polymerase.
2. DTT is required for stabilization of some enzymes.
3. RNasin is an RNase inhibitor.
4. One key to successful RT-PCR is the quality of the starting RNA. Because RNA is easily degraded once it is in solution, RT-PCR is best performed on samples recently extracted.

Table 3
Composition of PCR Reaction Mixes

Oligo mix for t(2;5)		Final concentration
Oligos (2;5)5′A and (2;5)3′A	0.075 µg of each/reaction	0.10–0.15 µ*M*
5X Buffer (pH 9.0) (Invitrogen)	16 µL/reaction	lX
DMSO	5 µL/reaction	5%
Taq polymerase (5 U/µL)	0.5 µL/reaction	0.025 U/µL
DEPC-H_2O	Make up to 80 µL/reaction	
Oligo mix for *NPM* controls		**Final concentration**
Oligos (2;5)5′A and *NPM* 3′A	0.075 µg of each/reaction	0.10–0.15 µ*M*
10X Buffer (Perkin-Elmer Cetus)	8 µL/reaction	1X
Taq polymerase (5 U/µL)	0.5 µL/reaction	0.025 U/µL
DEPC-H_2O	Make up to 80 µL/reaction	

Table 4
Composition of Nesting PCR Reaction Mix

Nesting oligo mix		Final concentration
Oligos (2;5)5′B and (2;5)3′B	0.075 µg of each/reaction	0.10–0.15 µ*M*
dNTPs (1.25 m*M* stock)	2 µL/reaction	25 µ*M*
l0X Buffer (Perkin-Elmer Cetus)	8 µL/reaction	1X
DMSO	5 µL/reaction	5%
Taq polymerase (5 U/µL)	0.5 µL/reaction	0.025 U/µL
DEPC-H_2O	Make up to 80 µL/reaction	

5. Due to degradation of RNA during tissue fixation and processing, it may be difficult to get good RNA from samples that have been formalin-fixed and paraffin-embedded.
6. As with any PCR procedure, contamination is always possible. As mentioned before (p. 166), single-run aliquots of reagents should be used along with dedicated pipettors. A separate set of pipettors should be used for nesting reactions.

Table 5
Touchdown PCR Program for *NPM-ALK* and Normal *NPM* Control Amplification

Denaturation	Annealing	Extension	Number of cycles
94°C/1′15″			X1
94°C/1′	65°C/1′	72°C/1′	X1
94°C/30″	64°C/1′	72°C/1′	X1
94°C/30″	63°C/1′	72°C/1′	X1
94°C/30″	62°C/1′	72°C/1′	X1
94°C/30″	61°C/1′	72°C/1′	X1
94°C/30″	60°C/1′	72°C/1′	X1
94°C/30″	59°C/1′	72°C/1′	X1
94°C/30″	58°C/1′	72°C/1′	X32
94°C/30″	58°C/1′	72°C/7′	X1

7. Random hexamer primers (Pharmacia, Piscataway, NJ) may be used at a final concentration of 5 pmol/μL for the RT reaction instead of the (2;5)3′A and *NPM*3′A oligo primers, if desired, with good result.
8. Although the bands should be visible with ethidium bromide staining, it is important to do a Southern transfer of the gel onto a nitrocellulose or nylon membrane, then hybridize with the *NPM-ALK* junction-specific probe in order to definitively establish the identity of the PCR products.
9. Signals should be of sufficient intensity to give a good autoradiograph following 4 h of exposure, although longer exposure is sometimes required.
10. If no signals are seen following PCR, the following steps should be taken:
    a. If no bands are present in the *NPM* controls, the RNA may not be clean enough to amplify. To clean, extract with phenol/chloroform/isoamyl alcohol, followed by extraction with chloroform/isoamyl alcohol, then precipitate the aqueous phase in NaOAc and ethanol.
    b. A second explanation for the lack of visible bands on an ethidium-stained gel is that one or more reagents are not good. RT and PCR reagents that are freeze-thawed multiple times (which should not occur with single-use aliquots), or that are stored in a freezer that does not maintain constant temperature, may go bad.
11. If the bands seen are weak, or many additional bands are seen, it may be necessary to optimize the PCR conditions for your own use. Key factors for amplification by PCR appear to be pH of the buffer; Mg^{2+}, dNTP, primer, and *Taq* polymerase concentrations; presence or absence of a cosolvent such as DMSO; and the cycling parameters.
12. If the bands on the gel look good, but there is no signal in Southern hybridizations, check the specific activities of the probes being used. A band of altered size may be seen in the rare cases of t(2;5)-positive NHL that possess variant

NPM-ALK fusion junctions ***(6,25)***. The *NPM-ALK* product generated from these samples will not hybridize to oligo (2;5)-P, which is homologous to *NPM* and *ALK* sequences present at the usual fusion junction. Such variant fusions may be detectable in hybridizations using one of the two nesting oligos as probes, depending on the location of the fusion junctions in *NPM* and *ALK*.

13. As with the t(14;18) and t(9;22), the t(2;5) — as detected by *NPM-ALK* RT-PCR — has been reported to occur at a low frequency in the peripheral blood cells of apparently healthy individuals, presumably reflecting the belief that the complicated rearrangement machinery that lymphocytes use to generate antibody and T-cell receptor diversity makes these cells more prone to "random" translocations ***(45)***. Thus, the results of *NPM-ALK* RT-PCR must be evaluated together with the clinicopathologic context in those instances in which the assay is unexpectedly positive.

Acknowledgments

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3

Flow Cytometry

9

Diagnostic Flow Cytometry in Hematologic Malignancies

Gerald E. Marti, Maryalice Stetler-Stevenson, and Thomas Fleisher

1. Introduction

1.1. Background

During the past 30 years, we have seen flow cytometry (FCM) emerge from being a research tool requiring a group of engineers, an optical bench, and a darkened room to a benchtop flow cytometer that is used routinely in a clinical setting. The flow cytometer is to cell biology what the UV-visible spectrophotometer is to solution spectroscopy. Several events have contributed to the development of this technology to where it is now an indispensable tool in the diagnosis and clinical monitoring of disease. Many cell types are now under intense investigation. The clinical application of FCM to lymphocyte subset immunophenotyping in the leukemias and lymphomas was responsible for the early development of clinical FCM.

The emergence of FCM as a widely used clinical laboratory tool in characterizing lymphocytes is the direct result of several recent developments. First, hybridoma technology has generated a large array of monoclonal antibodies directed at a variety of cell surface antigens, cytoplasmic and nuclear receptors. A second advance concerns the discovery of various growth factors (cytokines) and their receptors which has clarified cell development and growth. The molecular revolution in biology has been critical in determining the differentiation and developmental pathways of lymphocyte subsets.

A fourth area relates to advances in fluorochrome chemistry. One of the major driving forces in the formation of useful fluorochrome conjugates has been the development of suitable fluorochromes that are excited by a single wavelength. Adding to the arsenal of fluorochromes such as fluorescein

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isothiocyanate (FITC), phycoerythrin (PE), and peridin-chlorophyl-A-protein (PerCP) are the tandem conjugates. These consist of an acceptor fluorochrome conjugated to a donor fluorochrome, and when the donor fluorochrome (e.g., PE) is excited, its emission is coupled or transferred to the acceptor fluorochrome (e.g., Texas Red or one of the indodicarbocyanine [Cy5] derivatives). This coupling is carried out via fluorescence resonance energy transfer (FRET).

Development and refinement in instrumentation has also been important. Perhaps two of the most important developments have been the ability to use air-cooled lasers and the development of rigid or fixed optical systems. The latter being combined with a quartz flow cell that uses an aqueous polymer for optical collection of the emitted signals. Equally as important to hardware developments have been the developments made in software. User-friendly programs for the analysis of the vast amount of data that can be collected in FCM is an absolute must.

In addition to these developments in the areas of monoclonal antibodies (mAbs), cellular and molecular biology, fluorochromes, hardware, and software, there have been novel clinical situations or new challenges in medicine that have further propelled the use of this methodology. As noted, the immunophenotyping of leukemias and lymphomas has evolved to the point where leukemias and lymphomas are routinely evaluated using FCM in addition to routine and specialized slide-based technologies. In fact, FCM may be replacing cytochemistries, particularly terminal deoxynucleotidyl transferase (Tdt) and myeloperoxidase (MPO) assays. However, the HIV pandemic with the emergence of the absolute CD4 count as a prognostic marker did much to propel clinical FCM to the next stage.

The third clinical application that is emerging involves the absolute enumeration of CD34+ stem cells. At the same time, FCM is beginning to impact on transplantation in the setting of a flow-based crossmatch. Responses to therapy defined by apoptosis can now be measured by FCM. Granulocytic, lymphoid, and platelet function in terms of activation and cytokine profiles can also be determined by FCM. One would predict that the next phase of FCM will be an extension of the third stage involving the combined use of analysis and high-speed cell sorting for the detection of rare, metastastic tumor cells. In fact, we predict the emergence of high-speed cell sorting using a desktop flow cytometer equipped with three lasers and several PMTs. In the same vein, the recent use of FCM to detect dendritic cells and antigen-specific T cells further demonstrate the power of this technology to detect and resolve small cell subpopulations of interest.

In addition to lymphocyte immunophenotyping, it is important to realize that FCM is useful in identifying antigens associated with granulocytes, mono-

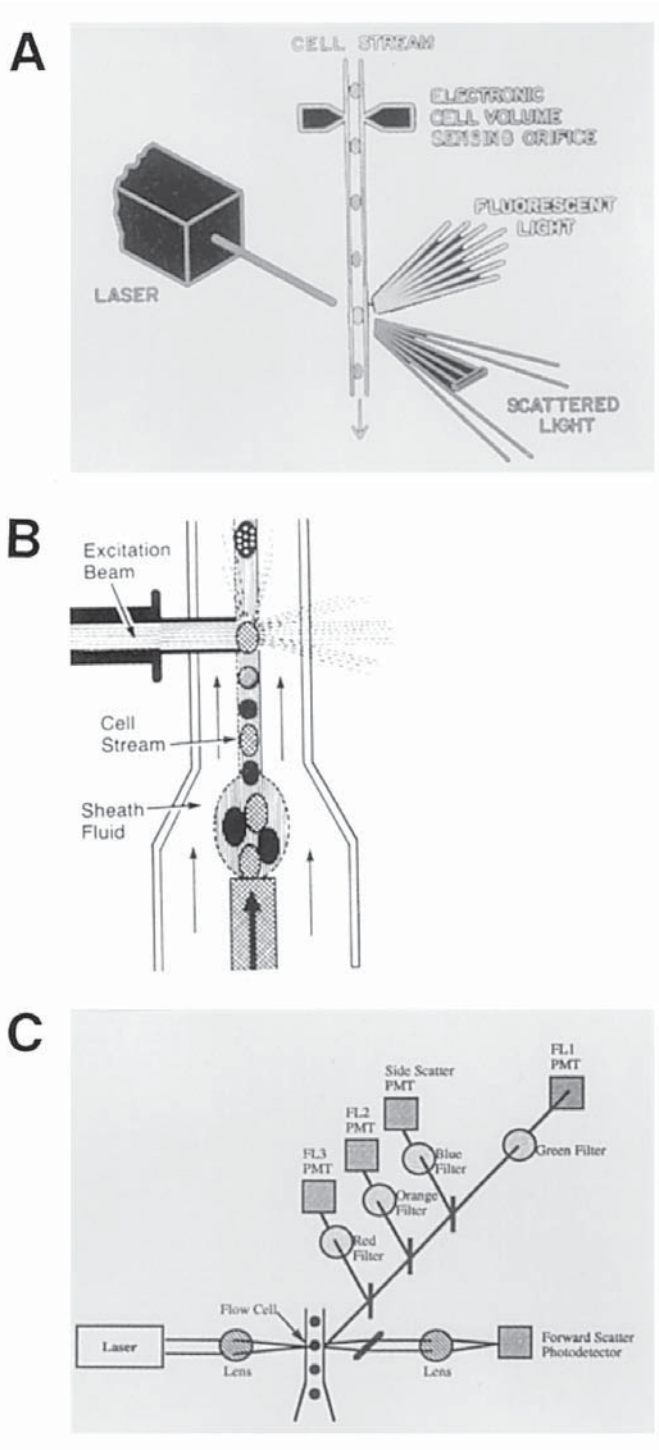
cytes, and other nonhematological cells. In fact, FCM is proving to be extremely useful in the area of nonneoplastic hematology, i.e., platelet activation, reticulocyte enumeration, and evaluation of red cell antigens, particularly those involved in such clinical entities as paroxysmal nocturnal hemoglobinuria (PNH). With this introduction to the clinical and investigative utility of FCM, we now turn our attention to a description of the instrumentation itself. An excellent introduction to these aspects of FCM can be found in the texts by Melamed et al. and Shapiro ***(1–5)***.

1.2. General Concepts in Flow Cytometry

1.2.1.1. Instrumentation

FCM is a rapid, dynamic method of multiparameter, multicolor, single-cell analysis that may be combined with cell sorting. In its simplest form, a suspension of cells is labeled directly or indirectly with a fluorescent probe. Next, the cells in the sample stream and surrounding sheath fluid are hydrodynamically focused into a coaxial jet stream. As the jet stream intersects the laser beam, fluorescent excitation can occur. Each cell is measured for light scatter signals in the presence or absence of emitted fluorescence. The laser beam, jet stream, light scatter, and fluorescence detectors (photomultiplier tubes) are precisely aligned orthogonally, i.e., they are all at right angles to one another (*see* **Fig. 1A** and **1B**).

The basic components of a flow cytometer are shown in **Fig. 1C**, **1D**, and **1E** and include the source of illumination, optical bench, fluidic system, optical axis and detectors, electronics, and computer. The source of illumination in a standard single-beam clinical instrument comes from a monochromatic light source, usually an air-cooled argon laser (488 nm wavelength). The optical bench contains lenses that focus and shape the illumination beam into a defined size to provide an optimal area of illumination. The point at which the light illuminates each single cell (particle), referred to as the point of interrogation (analysis), results in scattering of the incident beam and emission of fluorescent light. The various signals (scattered light and fluorescence) are then collected through appropriately arranged filters and photodetectors (photodiodes and photomultiplier tubes), and analog signals are digitized and sent to a computer for processing. This light (photons) impinges on a photomultiplier tube generating photon electrons. The output of this is further amplified and digitized. There are important considerations in terms of light collection efficiency, background signal, and the construction of filters and their proper use that are beyond the scope of this chapter (*see* **refs. *6–8***). **Figure 1** contains a summary of figures to explain FCM.


A
CELL STREAM
ELECTRONIC
CELL VOLUME
SENSING ORIFICE
FLUORESCENT
LIGHT
LASER
SCATTERED
LIGHT
B
Excitation
Beam
Cell
Stream
Sheath
Fluid
C
Side Scatter
PMT
FL1
PMT
FL2
PMT
FL3
PMT
Blue
Filter
Green Filter
Orange
Filter
Red
Filter
Flow Cell
Laser
Lens
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Forward Scatter
Photodetector

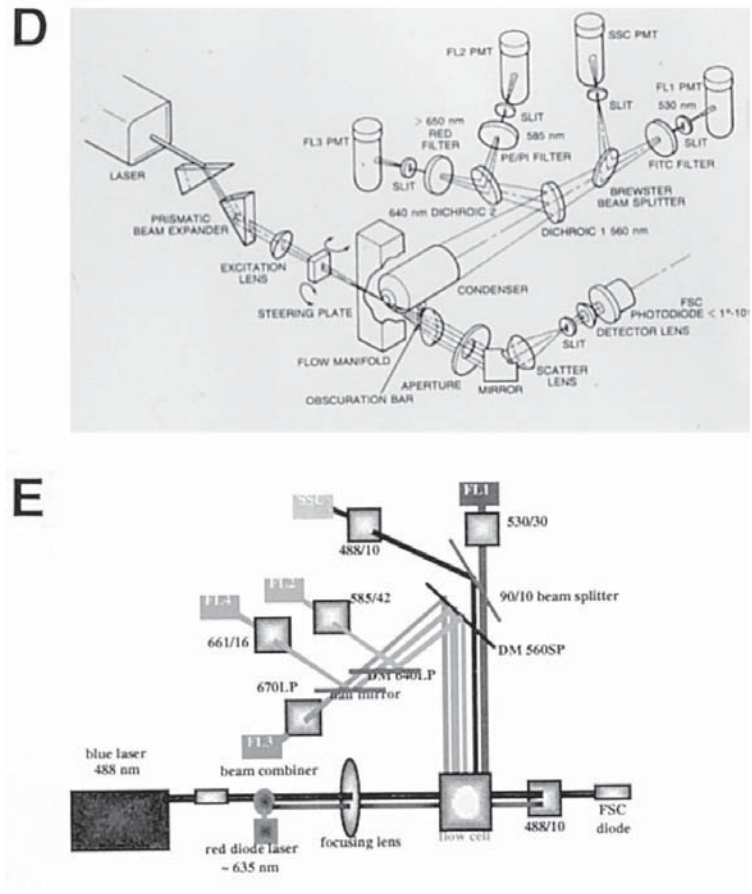


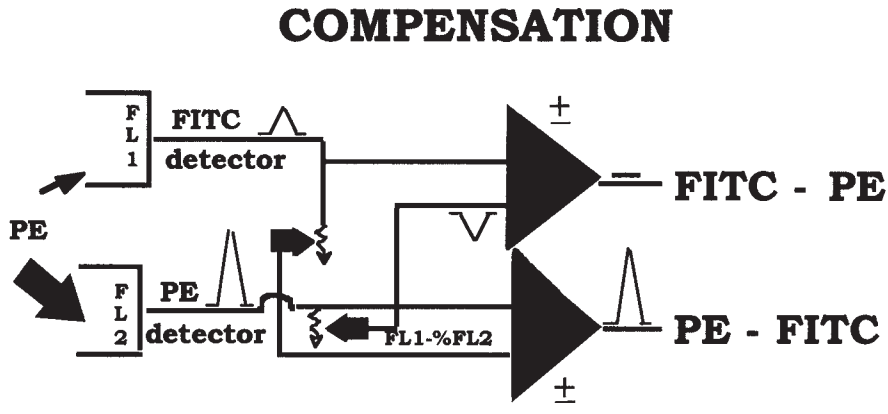
Fig. 1. Composite of selected slides. **(A)** Diagram of orthogonal alignment of laser, sample stream, and fluorescence detectors. **(B)** Diagram of single cells after hydrodynamic focusing. **(C)** Block diagram of flow cytometer components. **(D)** Schematic of actual components of a single-laser, three-color FCM. **(E)** Schematic of actual components of a dual-laser, four-color FCM.

1.2.1.2. Fluorochrome Reagents

Fluorochrome-conjugated mAbs are the routine probes used in FCM. Also, since the flow cytometer is equipped with a single argon laser emitting light at 488 nm, fluorochromes are selected that are suitable for excitation by 488. Examples of these include FITC-PE, PE-indodicarbocyanine tandem conjugates (PE-Cy5), propidium iodide (PI), and 7-amino-actinomycin D (7AAD). More recent reagents include allophycocyanin (APC) and PerCp and dimers of thiazole orange (TOTO-1).

Shapiro's textbooks ***(3–5)*** contain excellent summaries of these flurochromes with their optimal excitation and maximum emission wavelengths (e.g., third edition, Chapter 7, pp. 244–245) ***(5)***. The use of additional fluorochromes requires a second wavelength laser. This is usually a laser that provides monochromatic light at a higher wavelength for a fluoroprotein such as APC (*see* **Fig. 1E**). However, a UV laser has also proven useful. Of the reagents cited, FITC, PE, and PerCp are single fluorochromes and can be conjugated directly to antibody reagents. There is another class of fluorochrome compounds referred to as tandem conjugates that have been previously discussed (PE-Cy5). The excitation and emission of the PE molecule is transferred or coupled to the excitation and emission of the covalently linked Cy5 molecule. This results in a third color at a higher wavelength. It is important that the covalent linking chemistry be complete or there will be incomplete transfer of the light energy from the phycoerythrin to the Cy5, resulting in leakage of the phycoerythrin signal. As noted earlier, this process is also referred to as FRET and can be used to measure intermolecular distances of surface antigens ***(9)***.

Within the context of using two or more fluorochromes, the problem of fluorescence compensation must be addressed. Compensation is required when the fluorescence of one fluorochrome is detected in a second detector. This unwanted signal is due to spectral overlap and needs to be electronically removed (compensated). There are two approaches to this problem: one is in hardware and the other is in software. The hardware correction approach is also referred to as electronic-based subtraction compensation. In this method, a fixed amount of the output signal that varies as a function of photomultiplier tube (PMT) voltage is subtracted from either PMT. For two-color analysis, this approach is appropriate most of the time and can be conveniently checked experimentally by the use of single-stained cell preparations. When one goes to three or more colors, however, the number of potential subtractions that have to be performed and the required number of potentiometers results in unwanted electrical noise. Because of this, Shapiro has suggested an alternative approach to compensation that is based on a mathematical algorithm carried out in software (third edition, compare pp. 165 to p. 214) ***(5)***, but a comparisons of these methods has not been performed. These two principles of compensation are shown in **Fig. 2**. Compensation is not a trivial process and users are recommended to use compensation controls regularly. Many published two-parameter data sets are suboptimal because of inadequate compensation. A useful review of compensation can be found in the appendix of a recent paper by Stewart and Stewart based on using a combination of electronic and off-line software based compensation ***(10)***.



EQUATIONS WHICH MUST BE SOLVED FOR SOFTWARE FLUORESCENCE COMPENSATION

(after Bagwell CB, Adams EG: Fluorescence spectral overlap compensation for any number of flow cytometry parameters. Ann N Y Acad Sci 677:167-84, 1993)

$$F_1 = (1 - (k_{12} + k_{13} + k_{14}))f_1 + k_{21}f_2 + k_{31}f_3 + k_{41}f_4 + a_1$$

$$F_2 = k_{12}f_1 + (1 - (k_{21} + k_{23} + k_{24}))f_2 + k_{32}f_3 + k_{42}f_4 + a_2$$

$$F_3 = k_{13}f_1 + k_{23}f_3 + (1 - (k_{31} + k_{32} + k_{34}))f_3 + k_{43}f_4 + a_3$$

$$F_4 = k_{14}f_1 + k_{24}f_4 + k_{34}f_3 + (1 - (k_{41} + k_{42} + k_{43}))f_4 + a_4$$

where F_1 = measured value of fluorescence in band 1 (e.g., green fluorescence),
F_2 = measured value of fluorescence in band 2 (e.g., yellow fluorescence)
F_3 = measured value of fluorescence in band 3 (e.g., orangefluorescence)
F_4 = measured value of fluorescence in band 4 (e.g., red fluorescence)
f_1 = actual fluorescence emitted by label 1 (e.g., fluorescein fluorescence (in all bands))
f_2 = actual fluorescence emitted by label 2 (e.g., phycoerythrin fluorescence (in all bands))
f_3 = actual fluorescence emitted by label 3 (e.g., PE-Texas red fluorescence (in all bands))
f_4 = actual fluorescence emitted by label 4 (e.g., PE-Cy5 fluorescence (in all bands))
a_i = autofluorescence in band I
k_{ij} = fraction of fluorescence from label I in band j

Fig. 2. Approaches to the problem of spectral overlap. *upper panel*: Diagram of electronic compensation circuitry for subtraction method furnished by Carleton Steward. *Lower panel*: a mathematical description of the multiple equations needed to solve the compensation problem, which is copied from a slide from Howard Shairpo and is taken from **ref. *3***.

1.2.1.3. Sample Preparation

Sample processing and preparation are dependent on the sources, i.e., where and how they are collected, and whether cells or tissues are selected for analy-

sis. Blood is the most commonly analyzed biological material and this may be prepared by whole blood lysis, or as peripheral blood mononuclear cells [(PBMC) or peripheral blood lymphocytes (PBL)] from Hypaque-Ficoll density gradient centrifugation separation. A variety of apheresis and cell elutriation products are also available from peripheral blood. In addition to blood, one may examine bone marrow aspirates, lymph node suspensions, bladder washings, fine-needle aspirates, tissue culture cell lines, pollen samples, single-cell preparations of plant material, analysis of particulate matter from the sea, and even microbial organisms in the setting of germ warfare. However, we restrict our comments primarily to the use of whole blood.

Historically, Hypaque-Ficoll separation of PBMC/PBLs was used in immunophenotyping leukemias and lymphomas. However, this is a time-consuming procedure, and Fleisher et al. have shown selective loss of various lymphocyte subsets ***(11)*** and Fleisher et al. ***(12)*** and Marti et al. ***(13)*** have shown an enrichment of monocytes. The consensus conference on the flow cytometric immunophenotyping of leukemias and lymphomas strongly recommends that whole blood lysis be used in clinical settings ***(14)***.

Although the role of washing will be discussed subsequently, originally, there were two protocols: stain and lyse vs lyse and stain. Actually, the latter procedure was the first published procedure more than 10 yr ago. However, subsequently it was shown that staining followed by lysis was the preferred method (reviewed in ***[15]***). In the context of fluorescence intensity based on the work of Bossuyt et al., we have come to appreciate that essentially each reagent used in FCM needs to be evaluated in each given lysing system ***(16)***.

It is conventional to wash blood used in whole blood lysis preparations one or two times with centrifugation to pellet the cells. However, in the setting of a CD34 stem cell (progenitor cell) flow cytometric analysis, a wash and no lyse (WNL) procedure is often recommended ***(17,18)***. This relates to the observation that centrifugation may lead to a significant loss of cells with major impact on the accuracy of results when one is trying to measure rare events. Critical attention is not always given to the requirements of saturation, i.e., the temperature and concentration of antibody and the duration of the reaction. The impact of these variables on quantitative FCM is described in detail by Davis et al. ***(19)***. However, in the setting of standard immunophenotyping, this may not be so crucial. Equally important is the selection of mAb and fluorochrome configuration. To a certain degree, manufacturers select commercial antibodies for optimal affinity. Finally, in sample preparation one must give some attention to the fixative that is being used and how long the cells are permitted to remain in the fixative.

FCM has been utilized to determine absolute cell counts of specific subpopulations. This had been initiated with absolute CD4 lymphocyte counts in the setting of HIV. However, at that time historically, the absolute CD4 count was determined using a dual platform. This means that FCM was used to determine the relative percentage of CD4 cells and an automated hematology blood cell-counting device was used to determine the absolute lymphocyte count. When the FCM methodology for absolute CD34 counting was being developed, there was a desire to have a single flow cytometric platform for absolute CD34 counts. Therefore, counting beads were introduced. The principle of absolute counting is based on counting a known number of beads in a given volume. If the beads are at a concentration of 10, 100, or 1000 beads/μL, the number of cells counted simultaneously during the same time that 10, 100, or 1000 beads are enumerated is the concentration of the cells. The combination of counting beads, whole blood lysis, and the "no-wash" technique allows for absolute counting of CD34-labeled cells on a single flow cytometric platform as has been described by Marti et. al and Gratama et al. ***(17,18)***.

Intracytoplasmic antigens and cytokines can now be measured by adding a permealization step prior to staining that may affect the fixative used ***(20)***. DNA analysis typically requires the fixation of cells in cold 70% ethanol followed by RNA digestion and staining with propidium iodide (PI). Proprietary reagents are available to permealize and fix cells prior to staining for intracytoplasmic antigens such as kappa and lambda light chains and CD3 and so on. The recent interest in the flow cytometric analysis of apoptosis has also been combined with the incorporation of Brdu as a measure of cell proliferation. This is based on the Tdt-catalyzed incorporation of labeled or biotinylated dUTP into double-stranded DNA break sites. In vivo incorporated Brdu is detected with a second color. Thus, cell death and proliferation can be measured simultaneously, and this has been applied extensively by Darzynkiewicz et al. ***(21)***.

The kappa-lambda analysis particularly in the setting of chronic lymphocytic leukemia (CLL) deserves special consideration. This is a difficult assay requiring the use of multiple sets and combinations of anti-light-chain reagents to determine the presence or absence of monoclonal excess. For B-cell malignancies, the serological diagnosis of monoclonality is of central importance and remains as sensitive as polymerase chain reaction (PCR)-based methods, particularly when combined with a three-color analysis ***(22–24)***. The problem with the kappa-lambda analysis in BCLL stems from two observations: the decreased density of surface immunoglobin (Ig) on BCLL lymphocytes and the nonspecific binding of Ig to lymphocytes. Because of this, it is often diffi-

cult to determine which light chain is in excess or whether there is dual expression or absence of both light chains ***(25)***.

In a series of experiments, Stetler-Stevenson and colleagues showed that a simple step of washing was sufficient to remove cytophilic antibody selectively from B cells ***(22)***. Previously we had carried out cytoshedding at 37°C in bovine serum for 45 min (G.E.M., unpublished observation). For a period of time, the K-S test was advocated as a means of resolving small kappa-lambda differences but it does not appear to be widely used at this time ***(26,27)***. The current practice is to identify abnormal B cells based on size and or abnormal antigen expression (e.g., CD5+, dim or bright CD20) and then to determine light-chain expression within this abnormal group. This allows identification of minor subpopulations of monoclonal B cells, even within a predominantly polyclonal B-cell background. Lymphocytes in some samples of whole blood, particularly samples collected in ethylenediaminetetraacetic acid (EDTA), show an increase of double-labeled cells. This appears to be mediated by Ig, and can be transferred passively and eliminated by washing ***(28)***.

1.2.1.4. Data Acquisition

Over the years, we have come to appreciate the need for a unified setup and manufacturers have generated programs that qualify the instrument according to the manufacturer's recommendations. These require reference microbead standards. A reference microbead standard is characterized by a level of fluorescence intensity (FI) that is being measured on the cell of interest. More recently, a combination of a reference microbead standard with a set of calibration microbead standards and a certified blank has been developed. This permits one to select a point in sample space that will bracket the range of the scale that one wishes to measure. Once a reference point is determined, it may be validated with the use of calibration microbead standards. Such calibration curves have a linear range with deviations at both ends of the curve. The linear range permits the extrapolation of the molecular equivalents of soluble fluorochorme (MESF) or antibody binding capacity (ABC) of cells. More importantly such calibration curves permit one to establish and ensure that the instrument is performing according to a set of primary performance characteristics. Presently, this approach should also include a cellular or microbead compensation control. However, as noted earlier, the ability to do compensations in software has become more common. This procedure requires that each color be acquired and analyzed separately.

Several years ago we used the term quantitative flow cytometry (QFCM) in conjunction with the development of a quality control/quality control (QC/QA) program. When we started, the only available standard was fluoresceinated calf thymus nuclei ***(29)***. Today there are several sources of microbead stan-

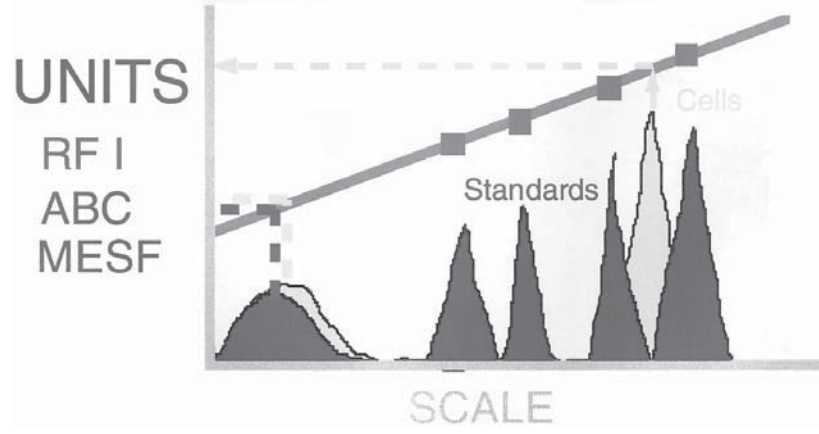


Fig. 3. Idealized calibration curve. The microbead standards consist of a blank bead (red peak to the left) and four levels of increasing fluorescence intensity (FI, also shown in red). The median channel log histogram channel number values are plotted on the *x* axis against their known level of FI. The calibration curve is obtained by regression analysis. The left-sided yellow peak represents unstained cells and or nonspecific binding. The yellow peak to the right represents the FI of the stained cells. Using the calibration curve, one is able to determine the number of units expressed on the cells. The goodness of fit between the microbead standards and the calibration curve is determined by the average percent residuals. The slope of the line is called the coefficient of response and is used to calculate the number of channels per decade and the number of decades in the given log amplifier. The negative bead is used to determine the threshold sensitivity. These determinations constitute the primary performance characteristics of a given flow cytometer.

dards. An example of the type of calibration curve obtained by using this method is shown **Fig. 3**. A protocol for the proper use of these calibration standards has been published by Schwartz et al. ***(30)*** and has been recommended by a National Institutes of Health (NIH) consensus conference ***(14)***. This method can be used to estimate the number of antibodies bound to a cell and to determine the number of antigen receptors on a cell. Zenger et al. have defined interlaboratory differences using this approach ***(31)***.

Once the instrument is set up according to the chosen method, samples may be analyzed with a confidence that is directly related to the standardized setup used. Routinely 10,000–20,000 cells are collected. For rare populations such as the CD34 stem cell subpopulation, 50,000–100,000 cells are collected, as an adequate number of the cells of interest must be collected. Therefore, in a patient with a B-cell neoplasm, collection of 1000 B cell events is a more useful goal than simply the overall number. On occasion, when analyzing for minimal residual disease, collection of up to 1 million events may be necessary that

require the preparation of multiple tubes for data acquisition. The exact number of cells that need to be collected to achieve statistical significance can be determined both theoretically and in practice. A minimum of 100 CD34+ cells is required to form a cluster for an accurate determination in unmobilized whole blood *(32)*. This would extrapolate to 5 or less CD34 cells/μL.

The majority of surface immunophenotyping data is collected using a log amplifier. The emitted photons are detected as photon-electrons amplified and digitized. These data may be digitized using 255 or 1024 channels of resolution. The default setting is usually at the level of 255 channels. It is worth noting that the log histogram channel numbers are routinely converted to relative fluorescence units. Many operators and investigators are unaware that data are collected on one scale and displayed on another scale. To further confuse matters, log scales and their conversions are not identical among manufacturers. Once the data file has been collected and stored, subsequent analysis can be done at a later date off-line. It should be noted that a standard in file formatting is necessary if one wants to analyze the data using different software packages. The Data File Standards Committee of the International Society for Analytical Cytology (ISAC) has published a Data File Standard for Flow Cytometry, Version FCS3.0 *(33)*.

1.2.1.5. Data Analysis

During sample analysis, flow cytometric data are often collected and saved in a LISTMODE file format. The LISTMODE data file consists of the correlated data parameters for each individual cell. For 100,000 events, such a file would consist of 100,000 rows and a column for each parameter collected. Typically, this would consist of two scatter parameters: forward light scatter and side scatter, and three fluorescent parameters: FL1, FL2, and FL3. The first column would contain the serial number of the cell being analyzed with the subsequent five correlated parameters in columns 2–6. These data are the numerical value of the amplitude for each parameter for a given cell or event.

The most basic data display form in FCM is the single-parameter histogram where the list mode data are combined or binned, i.e., the number of events or cells for the same channel will be combined. This is a population distribution display with the magnitude of the signal being measured on the *x*-axis and the number of events on the *y*-axis. Examples of this are shown in **Fig. 4**. Single-parameter histograms have the disadvantage of only allowing analysis of one parameter and therefore comparisons between two cell features cannot be made. In addition to single-parameter histograms, there are also two-parameter or bivariant histogram plots. In this display, any two parameters can be plotted against one another. This practice is very useful because it usually results in the detection or resolution of subpopulations suggested but not well resolved

or not appreciated in single-parameter histograms. This system of display allows the correlation of two different parameters simultaneously. In a system with two light-scatter parameters and one fluorescent signal, one would have the following bivariant plots: forward scatter-by-side scatter, side scatter by FL1, and forward scatter by FL1. With two fluorescent parameters and two scatter parameters, one would then have six bivariant comparisons. It is unfortunate that even with modern-day computers and displays, most of these types of data comparison are not made in the usual FCM laboratory, even though they are available and crucial to accurate data interpretation. **Figure 4** summarizes of common data displays used in FCM.

Two-parameter histograms can be displayed in several different ways including dot plots, contour plots, and isometric displays. In addition, there has been the appearance of contour density plots, based on the addition of color to dot plots. They may also be displayed as colored density contour plots. These can be very useful but require a color printer. However, the ability to correlate or follow color-coded subpopulations of cells in the various bivariant plots represents the heart of analysis in FCM. Also, with regards to the contouring algorithms, it is important to note that there are several different ways of producing contours: equally spaced linear levels, percentage of peak, and log percent of peak plots ***(34–36)***. Our experience suggests that the best contour plot option is to set the display level at the lowest possible number of cells. The detection level may be modified when you want to show that separating subpopulations requires plotting large numbers of cells. It is important to note that not all available contouring software programs are the same. Three-dimensional isometric displays are available and appear to be most useful in the reanalysis of enriched subpopulations. Advanced data analysis, which is beyond the level of this review, involves cluster analysis and artificial intelligence applied to the analysis of lymphocytes ***(37)***.

Under the topic of data analysis, one needs to address the issue of gating and gating strategies. Gating is a method to identify the cells of interest in a mixed cell population. Historically, this was aimed at resolving dead cells from live cells (so-called "live-dead discrimination") on the basis of a single scatter signal and a single fluorescent signal. The reason for live-dead cell discrimination lies in the fact that dead cells often bind reagents as well as live cells. The use of two-parameter light scatter gating evolved when both forward scatter and 90° light scatter became available on the same instrument. With the advent of whole blood lysis, it became necessary to identify and resolve different leucocyte populations. Forward vs side scatter plots allow differentiation of granulocytes, monocytes and lymphocytes into discrete populations in normal peripheral blood. This was used to gate on lymphocytes with the exclusion of other cellular elements and was soon referred to as "lymphocyte scatter gates" or "lymphocyte gates."

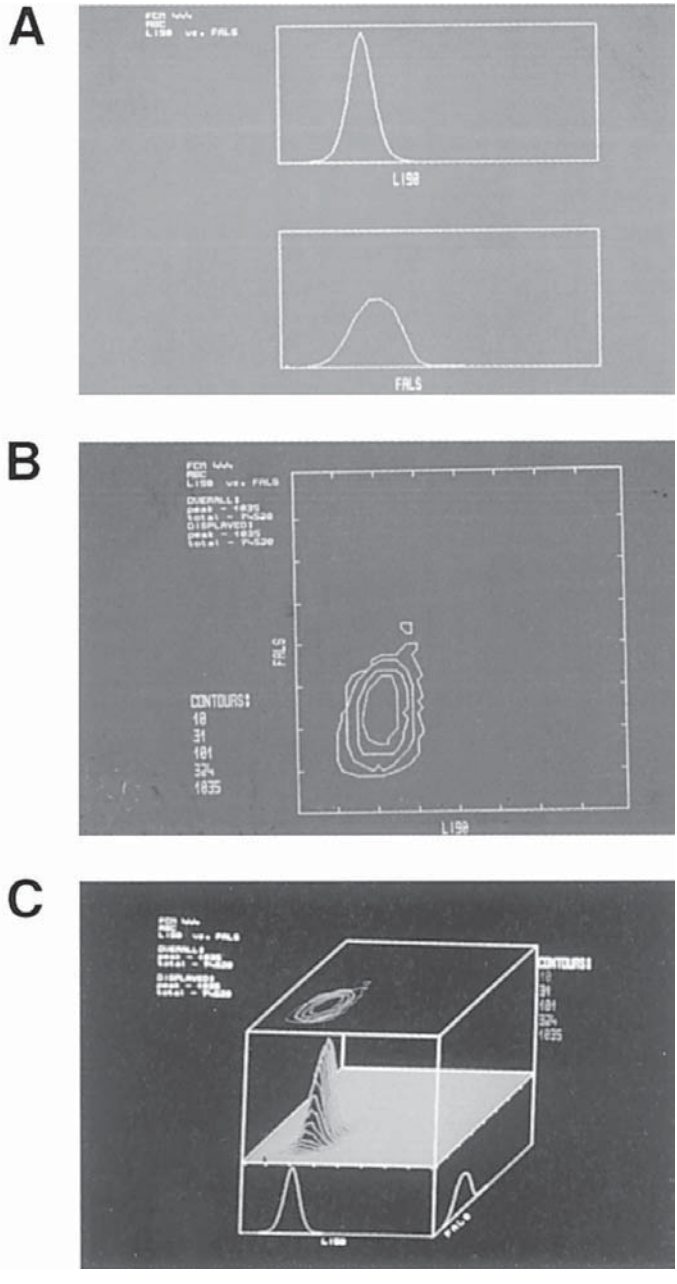


Fig. 4. Selected data displays. **(A)** Two single-parameter histograms. **(B)** The same two single-parameter histograms plotted as a bivariant plot. The small isolated contour is thought to be reticulcytes. **(C)** A three-dimensional display of same data in **(B)**. **(D)** Contour plots of platelets, red cells, lymphocytes, monocytes, and granulocytes. **(E)** An isometric display of the same data in **(D)**.

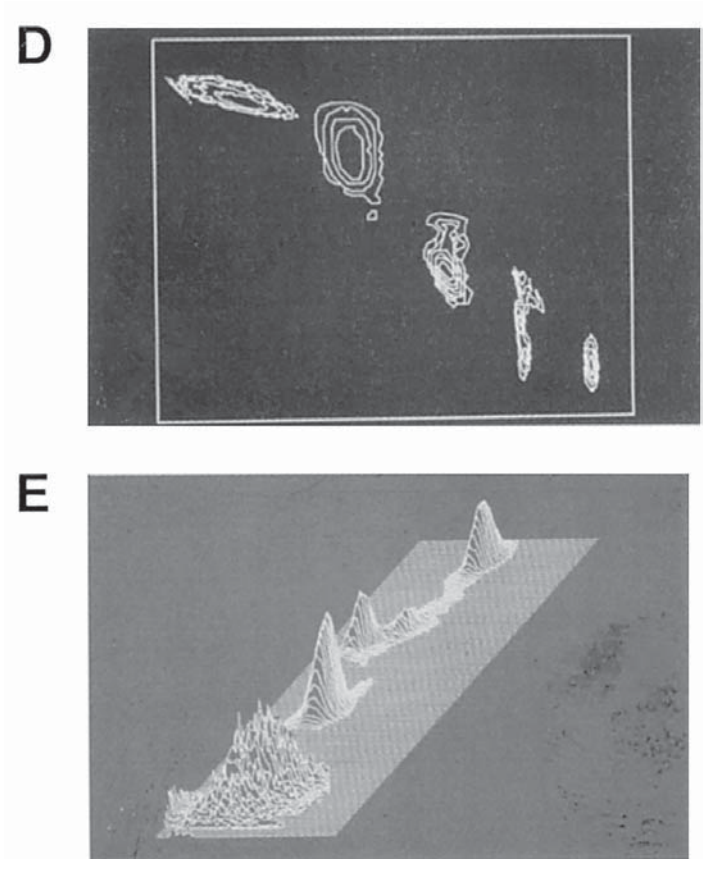


Fig. 4. *(continued)*

It was subsequently noted that the previously "tight lymphocyte gates" used for the analysis of gradient density separated PBMC/PBL inadvertently excluded some lymphocyte cells from analysis. This led to the use of a gating reagent and the concept of backgating to determine where the lymphocytes were located and to reestablish wider lymphocyte scatter gates ***(38)***. This was a very fortuitous development in FCM, for it resulted not only in a correction in our concept of lymphocyte gating, but it resulted in the fluorescent definition of lymphocytes in particular and leukocyte populations in general. The definition of lymphocytes as bright CD45, negative CD14 granulocytes as dim CD45, intermediate CD14 and monocytes as bright CD45, bright CD14 was a significant advance in better discrimination of lymphocytes. Proper standardization allows generation of a three-part differential that is equal to or superior to conventional automated blood cell counter determinations. Several examples of gating are shown in **Fig. 5**.

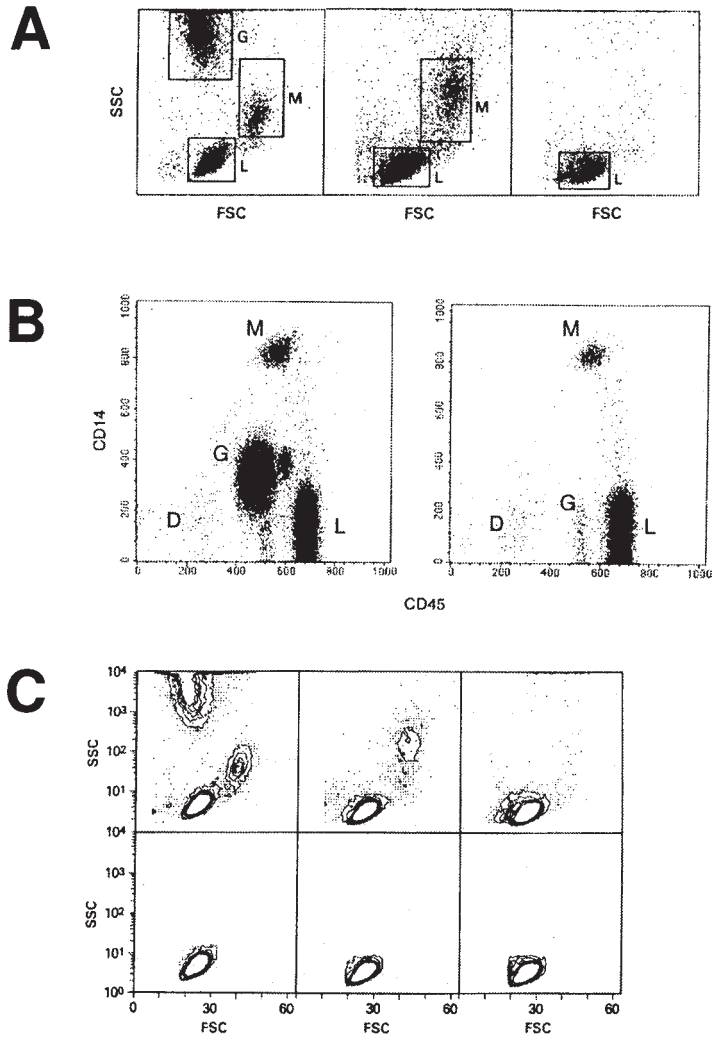


Fig. 5. Selected examples of gating. **(A)** Dot plots of whole blood, Hypaque Ficoll separated PBLs, and monocyte-depleted PBL. **(B)** Whole blood with and without a gate showing the variation in CD45 and CD14 staining. **(C)** The same data shown in **(A)** as contour plots with and without out gates. **(D)** The corresponding CD45/CD14 plots of stained cells based on scatter in **(C)**. **(E)** The effect of no gates (upper plots) and the presence of gates (lower plots for B and T cells. **(F)**. Representative plots of coexpression and mutual exclusion of selected antigens.

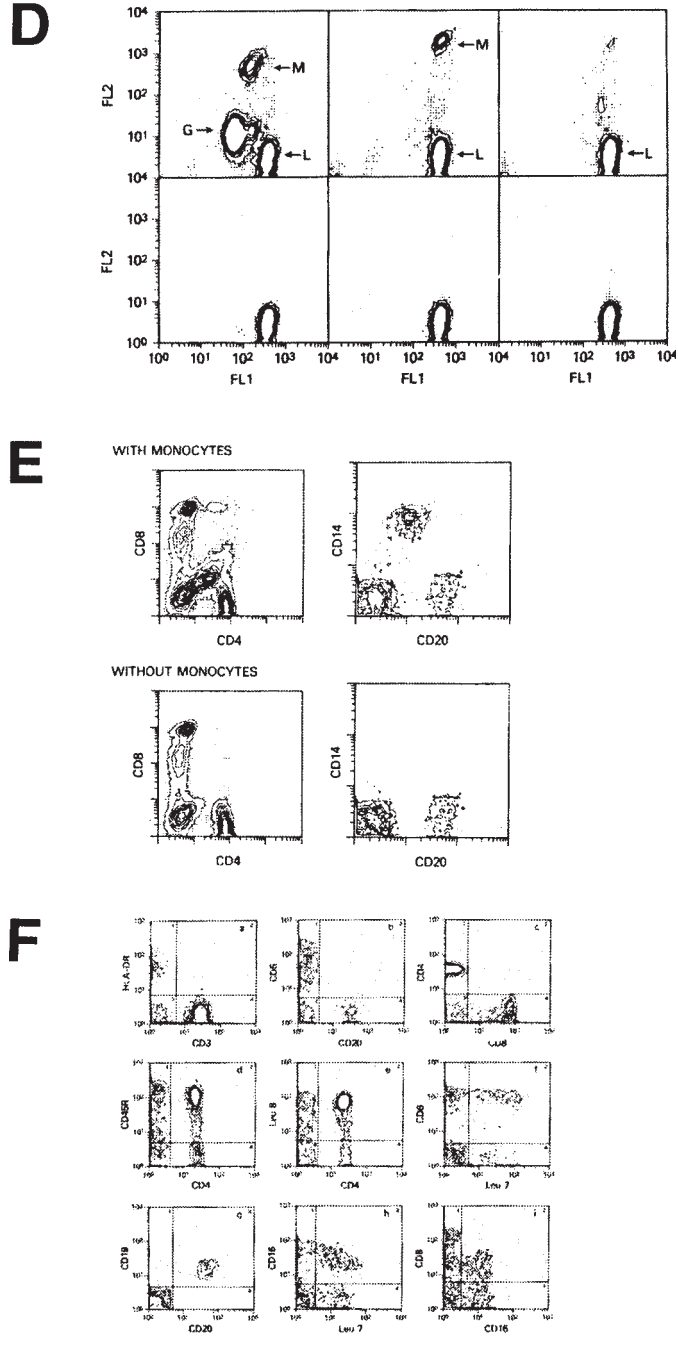


Fig. 5. *(continued)*

The use of fluorescent intensity to identify subpopulations of cells was explored in the evaluation of bone marrow aspirates using CD45 vs side scatter (SC.) for gating purposes. Myeloid and lymphoid blasts are not discriminated from erythroid precursors and mature lymphocytes based upon forward scatter and SC. Thus scatter-based analysis gates do not allow for an accurate determination of the immunophenotype of leukemic blasts. However, a plot of CD45 fluorescence intensity vs SC separates the bone marrow elements into the following categories: lymphocytes (bright CD45, low SC), monocytes (bright CD45, moderate SC.), granulocytes (dim CD45, high SC.), blasts (dim CD45, low to moderate SC.), and erythroid precursors (negative CD45 and low SC.). **Figure 6** is a representative CD45 versus SC. bivariant plot of a normal bone marrow aspirate.

1.3. Clinical Flow Cytometry

1.3.1. Immunophenotyping Blood

Surface immunophenotyping of lymphocyte subsets is the most widely used multiparameter, flow cytometric, diagnostic test available today. The choice of immunophenotyping reagents depends on the cells being analyzed and the questions being asked. Many laboratories use the concept of panels defined as the set of reagents required to define a clinical disorder or groups of related disorders in terms of a recognizable pattern of immunophenotypes. A panel may be defined with as few as three or four reagents within a single tube. Most panels contain some redundancy, i.e., the same reagent in more than one tube. The use of a large general panel recommended by consensus to immunophenotype an unknown cell type is a useful practice in research and may save time in setting up a second panel because the cell of interest was not satisfactorily immunophenotyped the first time or is no longer available. However, this must be balanced with reagent cost, analysis time, and frequency of positive findings.

It is our practice to include gating, T-cell, B-cell, and natural killer (NK)-cell reagents in every panel. This approach provides an important internal control based on the principle that the whole is the sum of its parts: the total percentage of lymphocytes in the gate is determined by the gating reagent and should approximately equal the sum of the percentage of T cells, B cells, and NK cells (100% ± 10%). When this relationship does not hold, an explanation must be sought. It is generally appreciated that that total CD4 and CD8 T cells are determined as CD3/CD4 and CD3/CD8. This is a form of T-cell gating and ensures that the CD4 and CD8 subsets are determined accurately. Expression of specific antigens by neoplastic lymphoid cells may allow subclassification of the detected lymphoproliferative disorders if a large enough panel is used.

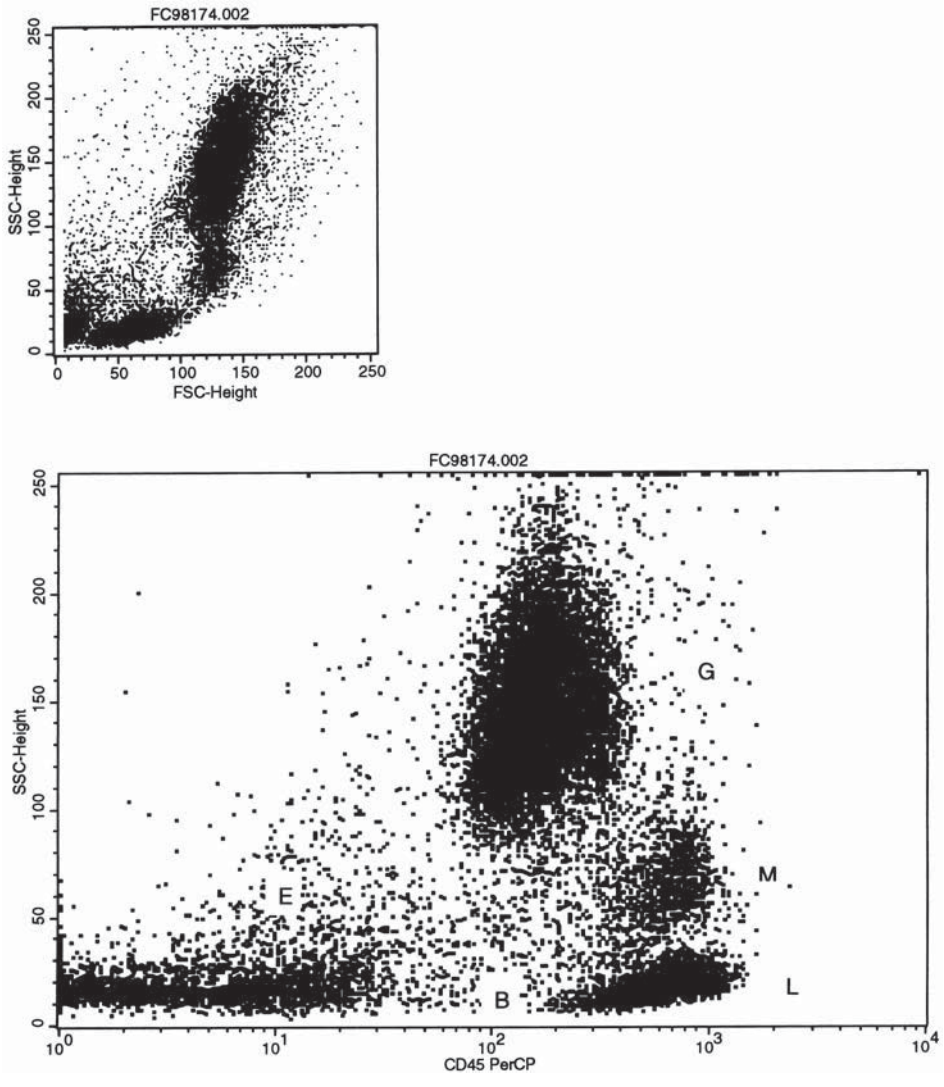


Fig. 6. A representation CD45 versus SC. bivariant plot of a normal bone marrow aspirate.

As this is vital clinical data, inclusion of antibodies allowing subclassification of tumor cells is highly recommended. Because one of our interests is in immunophenotyping sporadic and familial BCLL (G. E. M.), we design and use a panel in collaboration (M. S. S.) that not only confirms the presence of the BCLL clone but attempts to distinguish it from other closely related B-cell neoplasms.

Guidelines for immunophenotyping by FCM have been developed. Thus an expanded panel may also be useful in research. The recommended strategy for immunophenotyping has been developed by consensus in the setting of HIV. In addition, it should be noted that an advanced T-cell immunophenotyping procedure has been evaluated and published by the CDC. NIH U.S.-Canadian Guidelines for the Flow Cytometric Immunophenotyping of Leukemias and Lymphomas have been published ***(14)***. These guidelines discuss the indications for ordering testing and is important for laboratory and report standardization as well as data analysis. The guidelines point out the need to utilize a panel that will identify all possible abnormal cell populations within the specimen. For example, in the potential setting of the diagnosis of CLL, B-cell and T-cell lineage, as well as other low-grade lymphoproliferative diagnoses, must be considered in designing a panel instead of just studying so-called CLL antigens. Although it is beyond this review, it should be noted that under the rubric of cost containment, the Health Care Financing Agency (HCFA) is trying to determine the number of reagents that the government will approve for reimbursement.

1.3.2. Immunophenotyping Acute Leukemia

As noted in **Subheading 1.**, clinical FCM had its beginnings in the immunophenotyping of leukemias and lymphomas. The application of immunophenotyping to bone marrow aspirates is one of the most recent important advances in the clinical analysis of hematopoiesis. A diagnosis of an acute leukemia almost always involves the analysis of a bone marrow aspirate. The ability to resolve subpopulations in a marrow aspirate is not unlike the analysis of peripheral blood. It requires proper gating to be able to define the blast population and this is accomplished by plotting CD45 intensity on the *x*-axis and side scatter on the *y*-axis ***(39)***. Whole blood lysis is the optimal control sample to determine the location of the blast population in a marrow sample. In addition to pattern recognition, the second important aspect of marrow analysis is the control and standardization of fluorescence intensity. The surface markers on bone marrow cells are usually of lower intensity. Therefore standardization of fluorescence intensity measurements is of practical necessity. Jennings and Foon suggest that the flow cytometric analysis of acute and chronic leukemia is an interpretation that combines pattern recognition and fluorescence intensity measurements of antigen expression to reach a definitive diagnosis ***(40)***. It is also important to note that one of the underlying principles of diagnostic clinical FCM is the correlation and integration of morphologic, immunologic, and molecular cytogenetic data. The reason for this diagnostic approach is not only to define relevant biological subgroups but to also provide prognostic data for physicians, patients, and their families. A brief discussion of the major

hematologic malignancies follows. It is to be noted that flow cytometric immunophenotyping identifies the myeloid or lymphoid origin in 98% of the cases studied ***(40)***. Acute undifferentiated leukemia and biphenotypic leukemia will not be discussed.

1.3.2.1. Acute Lymphoblastic Leukemia (ALL)

Stetler-Stevenson et al. note that more than 90% of acute lymphoblastic leukemia and approximately 10% of lymphoblastic lymphomas are immature B-cell neoplasms ***(41)***. These precursor B-cell neoplasms do not express surface immunoglobulin and are characteristically Tdt positive. A minority of these cases expressed cytoplasmic immunoglobulin (IgM) and are considered to have a pre-B-cell immunophenotype. The remainder of cases express the CD10 antigen and Class II (HLA-DR) and historically were designated as having a common, null cell or a non-B, non-T immunophenotype. With the advent of multiple mAb specificities and gene rearrangement analysis, almost all cases of non-B-cell and non-T-cell ALL have been found to be precursor B-cell neoplasms. The majority of these neoplasms express the pan-B-cell antigen CD19, whereas approximately half of the cases expressed the pan-B-cell antigen CD22; CD20 is expressed less commonly. In contrast, approximately 10% of ALL and more than 90% of cases of lymphoblastic lymphoma (LBL) are immature T-cell neoplasms with immunophenotypes that correlate with an intrathymic stage of maturation. The majority of LBL and ALL also express the CD1, CD2, CD3, or CD5 antigens. In general, the cases of ALL are slightly less mature than cases of lymphoblastic lymphoma. Virtually all are either positive for Tdt and or the CD7 antigen, the earliest T-cell-specific antigen expressed. The majority (approximately 85%) either coexpress CD4 or CD8 or are negative for both. Neoplasms corresponding in differentiation to late cortical and medullary thymocytes are less common and are characterized by expression of either the CD4 or CD8 antigens.

Jennings and Foon ***(40)*** divide ALL into (1) B-precursor ALL, (2) pre-B ALL, (3) BALL, (4) TALL, and (5) infantile ALL. They further divide B-precursor ALL into CD10 positive or CD10 negative defining the two major subgroups. B-precursor-ALL is probably the earliest recognizable state in B-cell differentiation. It is characterized by HLA-DR (Class II) CD19, CD10, CD24, CD34, and Tdt positivity. The CD10-positive, B-precursor ALL has a more favorable prognosis than the CD10-negative group. B-precursor-ALL is, by definition, surface membrane immunoglobulin-negative.

Pre-B-cell ALL is characterized as HLA-DR+, CD19+, CD24+, CD9+, CD10+, CD34–, cytoplasmic immunoglobulin-positive but surface Ig negative. Tdt and CD20 expression is variable. By definition, this subgroup is characterized by the presence of cytoplasmic μ heavy chains and may be linked to

a cytogenetic abnormality; t(1;19), which may result in the creation of an X2A-PBX1 fusion product ***(40)***.

B-ALL is characterized as mature B-cell ALL and is equivalent to Burkitt's lymphoma in the leukemic phase. It is characterized by a mature phenotype consisting of surface Ig, HLA-DR, CD19, CD20, CD22, and CD24 expression but variable CD10 expression and absence of CD34 and Tdt. These patients characteristically have translocations involving the *c-myc* gene at 8q24 and either the heavy chain locus at 14q34, kappa light chains at 2p11, or lambda light chains at 22q11. This group of patients tend to have FAB-L3 morphology, whereas the other two groups of ALL patients have FAB L1 or L2 morphology.

The most common T-cell ALL immunophenotype consists of CD1, CD2, CD5, and dual CD4/CD8 expression with low-density CD3 expression, and Tdt is frequently positive. A pre-T-cell phenotype is characterized by CD7 positivity and cytoplasmic CD3 positivity with the absence of other T-cell antigens. T-cell neoplasms are characterized by the absence of specific normal T-cell antigens and/or display of aberrant combinations ***(41)***.

Infantile ALL with CD19-positive and CD10-negative but aberrant CD15 expression is likely to have a translocation involving 11q23 and a poor prognosis. Cases of ALL may be subclassified based on immunophenotypes and DNA content. Classification of ALL on the basis of DNA content results in two important subgroups: hyperdiploid cases with better prognosis and hypodiploid cases with poor prognosis.

It is worth noting that the pattern of expression of B-cell antigens is helpful in detecting normal hematogones. Maturing CD19- and CD10-positive B cells have a continuous spectrum of CD20 and surface Ig from negative to that of a mature B cell. The presence of hematogones defined as CD19-positive, CD10 B-precursors that are seen in a variety of clinical settings and most frequently after chemotherapy must be kept in mind so as not to misdiagnose relapsed or residual ALL. A recent study from Catovsky and colleagues describes a quantitative FCM method for distinguishing hematogones from residual ALL ***(42)***. Davis et al. have conducted a similar study in adult marrows after chemotherapy and note difficulty in distinguishing hematogones from residual marrow blasts ***(43)***.

1.3.2.2. Acute Myeloid Leukemia (AML)

The major aim of various consensus protocols in AML have been to define the immunophenotype of the malignant cells and to correlate this with classically defined neoplastic disease categories such as the French-American-British (FAB) subgroups ***(44)***. It should be emphasized that current evidence does not support routine flow cytometric immunophenotyping for the diagnosis of myeloid leukemias. However, Serke ***(44)*** notes that there are three FAB

AML subgroups in which immunophenotyping is essential for making the correct diagnosis: AML FAB 0 (undifferentiated leukemia), AML FAB 6 (erythroleukemia), and AML FAB 7 (megakaryocytic leukemia), which can be diagnosed only by means of immunological staining. In situations when morphology and cytochemistries are not clear, immunophenotyping may be required to diagnose other FAB subcategories (M.S.S. personal observation). Furthermore AML FAB 2 t(8;21) is diagnosed by the expression of the B-linkage associated antigen CD19 on these myeloid blasts. Serke concludes that the additional information obtained by multiparameter FCM allows a more subtle description of neoplastic cells ***(44)***. In addition, many hematology laboratories are doing away with slide-based cytochemistry studies such as MPO and Tdt assays that can be more easily performed by FCM.

Jennings and Foon recommend the original FAB criteria as modified by the National Cancer Institute-sponsored workshop that incorporates and correlates morphologic, immunologic, and cytogenetic data where appropriate ***(40,45,46)***. They define the immunophenotype of the following AML FAB subgroups:

1. M0 is described by blasts that are by definition cytochemically negative but express at least one myeloid marker such as CD13, CD33, or CD11B. Cytoplasmic MPO may be present and detected by mAbs. M0 blasts are typically positive for HLA-DR and CD34.
2. M1 blasts are characterized by CD13, CD33, and HLA-DR positivity. The level of CD34 expression may be less than M0 and there may be partial CD15 expression.
3. M2 is characterized by a reduced percentage of blasts sometimes referred to as AML with differentiation. CD34 is less prominent and CD15 is more prominent than M1, whereas most cases are HLA-DR positive. Expression of CD19, and less often CD56, in the setting of M2 is associated with the presence of t(8;21) as noted by Serke ***(44)***.
4. M3 is acute progranulocytic leukemia (APL), which may be either the hypergranular form or the microgranular variant (M3v). CD33 and low-density CD13 are usually present, whereas CD2 may be seen in the M3v, and HLA-DR is often negative in both forms. In fact, the presence of CD2 and HLA-DR negativity correlates with M3 associated with the t(15;17) translocation, which results in a rearrangement of the retinoic receptor-alpha (RAR-alpha) locus. A newly described entity, myeloid/natural killer cell acute leukemia, has a morphology and an immunophenotype also similar to M3 but without RAR-alpha rearrangements. It is seen in older patient populations, tends to be aggressive, and does not respond to all-trans-retinoic acid (ATRA). Although the morphological diagnosis of hypergranular AML M3 can be straightforward, the diagnosis of its hypogranular variant (M3v) is more difficult, making FCM is a useful adjunct in these cases.
5. M4 is myelomonocytic leukemia, whereas M5 is monocytic leukemia of which there is a recognized monoblastic variant. These two categories have similar

phenotypes with M4 expressing CD34 more often than M5. The combination of CD33 positivity with the absence of CD13 and CD34 is correlated with an M5 phenotype but occurs only in a minority of patients. Overall, the presence of CD13, CD33, HLA-DR, CD14, and CD15 are the characteristic immunophenotypes of M4 and M5. The presence of CD2 is correlated with an important subtype—M4EO (an eosinophilic variant).

6. M6, sometimes referred to as erythroleukemia, is best detected using antibodies directed toward glycophorin, which determines erythroid differentiation.
7. M7 (acute megakaryoblastic leukemia) can be detected with reagents specific for von Willebrand factor or can be identified with reagents to platelet antigens such as CD56 (GP3a) and/or CD41 (GP2b-3a). False-positive reactions may be due to platelet satellitism to leukemic blast have been noted in both blood and marrow samples.

A new classification scheme incorporating cytogenetics, immunophenotyping, and prognostic indicators is being developed by the World Health Organization (WHO). WHO is attempting to define diagnostic categories with predictable clinical course and response to treatment, i.e., a prognosis-based scheme ***(47)***.

1.3.3. Immunophenotyping Chronic Leukemia

1.3.3.1. Chronic Myelogenous Leukemia (CML)

In the chronic phase of this myeloproliferative disorder, FCM is of little use, whereas in the acute or blast-transformation stage it is very useful. FCM in the acute phase may be used to differentiate lymphoid from myeloid transformation. The lymphoid transformation usually involves a CD10-positive B-precursor ALL that is typically more responsive to chemotherapy than with myeloid transformation. A T-lymphoblastic transformation can occur and this frequently presents in the lymph nodes prior to bone marrow or blood involvement. There was an original enthusiasm for detecting multilineage transformation (mixed proliferations consisting of lymphoblasts, myeloblasts, erythroblasts, and megakaryoblasts) in the setting of blast transformation; however, this appears to have only limited clinical utility ***(49)***.

1.3.3.2. Chronic Lymphocytic Leukemia (CLL)

B-cell chronic lymphocytic leukemia (B-CLL) is closely related to other low-grade malignant lymphoproliferative disorders such as prolymphocytic leukemia (PLL), hairy cell leukemia (HCL), splenic lymphoma with villous lymphocytes, Waldenstrom's disease (immunocytoma), and the leukemic phase of small-cell lymphocytic, centrocytic, or follicular lymphoma and other non-Hodgkin's lymphomas. The differentiation of B-CLL from these other lymphoproliferative disorders can be made by a combination of cytomorphology and immunophenotyping (*see* **Table 1** for a summary).

Table 1
B-Cell Lymphoproliferative Disorders

Lympho-proliferative disorder	CD19	CD22	CD20	CD10	CD79a	sIg	CD5	CD23	CD11c	CD103	FMC7	CD25
CLL (M D+/–, G–/+)	+	+*	+*	–	+*	+*	+	+	–/+*	–	–	+
PLL	+	+	+^	–	+	+	–/+	–/+		–	+	
MCL (M, D)	+	+	+	–/+	+	+ (M/D)	+	–	–	–	+	+
Marginal zone (M > G/A)	+	+	+	–/+ mz	+	+	–	–/+	–/+*	–	+	–/+
FL (M > D > G > A)	+	+	+	+	+	+	–	–/+	–	–	+/–	+/–
HCL (M+/–D, G, or A)	+	+^	+^	–hcl	+	+	–	–/+	+^	+	+	+^
Immunocytoma (M)	+	+	+	–	+	+ (Mu)	–	–	–/+*	–	?+/–	–/+*

+ >90%, +/– >50%, –/+ < 50%, – <10%. MCL: cyclin D1 positive.
*Decreased expression.
^Increased expression.
Immuncytoma is cytoplasmic IgM by definition.
There is a lambda light-chain predomiance in MCL and HCL.
Abbreviations: CLL, chronic lymphocytic leukemia; PLL, prolymphocytic leukemia; FL, follicular lymphoma; HCL, hairy cell leukemia.

Melo and colleagues carried out several informative studies on the natural history of B-CLL ***(49–52)***. Morphologically, B-CLL may be subclassified as CLL/small lymphocytic lymphoma (SLL), CLL/PL, and CLL/mixed ***(53)***. CLL/SLL is the typical pattern seen and consists of cells that are larger than a red cell but smaller than the area occupied by two red cells. The N/C ratio is typically high >0.9, chromatin is clumped, nucleoli are not seen, and the nuclear contour is round without notching or clefts. SSL is used when the lymph node cytomorphology is the same but the leukemic involvement is less than 5000 cells/μL. CLL/PL is used to designate patients with a prolymphocyte fraction that varies from 10–55%. These cells are typically larger than the area of two red cells and have a lower N/C ratio, finer chromatin, and a conspicuous nucleolus. Notches and clefts are not prominent. CLL/MIXED is used to designate patients who have a component of larger cells that are not prolymphocytes. Even with these subclasses, individual patients still may show considerable morphologic heterogeneity on the basis of size. A variable portion of truly

small cells and lymphoplasmacytoid cells may also be admixed. This description is based primarily on blood films from untreated patients and does not involve the use of albumin preparations.

The typical immunophenotype in B-CLL can be characterized as a CD5+, CD19+, dim CD20+, dim CD22+, dim CD23+, CD79a+, sIg dim, and B-cell monoclonal lymphocytosis with light chain restriction ***(40,54)***. Jennings and Foon further note that CD43 is positive, and we would add CD38 ***(40)***. It is interesting that Jennings and Foon note that both CD10 and CD22 are negative. In our experience, CD22 is positive, whereas CD25 and CD11c tend to have a low level of expression. CD20 expression is characteristically reduced and, in conjunction with CD5 coexpression, creates a classic signature of this disease even when the absolute lymphocyte count is at the lower limit of normal ***(55–59)***. The earliest expressed antigen is the common CLL antigen ***(60)***. In our experience, these cells may also express CD11c and CD25 but are negative for CD10 and CD103. Expression of CD38 is also often seen and variant immunophenotypes expressing CD2 or CD8 are observed [personal observation and ***(61)***]. However, FCM7 is typically negative in B-CLL but positive in mantle cell lymphoma (MCL) and therefore is useful diagnostically. FCM7 positivity is common in the subclass CLL/PL.

Multiple cytogenetic abnormalities have been reported in BCLL with the most frequent being trisomy 12, and abnormalities of 11q, 13q, and 14q often in the form of deletions. Unfortunately, the immunophenotypes, morphology, and cytogenetic changes are not well correlated in B-CLL. Some feel that atypical B-CLL or CLL/mixed is associated with trisomy 12 ***(62)***. However, the exact distributions of the morphologic subtypes is not known and overlap would be expected. Criel et al. have attempted to bring some order into the subclassification of B-CLL ***(63)***. They note that typical CLL/SLL is monomorphic, whereas atypical B-CLL includes CLL/PL and CLL, mixed cell. They also note that CD79b is absent on CLL and the role of myelomonocytic markers and CD11c as they relate to morphology is unclear ***(64–68)***. In their morphological review of 390 cases of CLL selected out of a total of 418 cases, they conclude that typical and atypical B-CLL are two distinct but closely related clinical entities. Atypical CLL regardless of clinical stage is thought to be biologically a more aggressive disease. Of further interest, these investigators have correlated typical and atypical CLL with nodal patterns characterized by the presence or absence of paraimmunoblasts, prolymphocytes, pseudofollicles, and nuclear irregularities ***(69)***.

1.3.3.3. Large Granular Lymphocyte (LGL) and NK Disorders

LGL leukemias are clonal proliferations of mature cytotoxic lymphocytes characterized by increased numbers of circulating large granular lymphocytes

and chronic neutropenia ***(70)***. They have previously been referred to under a number of names, including T-g lymphoproliferative disorder and T-CLL ***(71,72)***. They can be divided into T-cell and NK-cell types. T-cell LGL leukemias typically present with recurrent bacterial infections due to neutropenia. Patients are usually middle aged (median 55 yr old), but the disease has been reported in patients aged 4–88 yr old. The characteristic findings are a modest lymphocytosis with increased T-cells expressing cytotoxic lymphocyte antigens (immunophenotype) as well as increased LGLs on the peripheral smear that are larger than normal in cell size and display abundant cytoplasm with prominent azurophilic granules. However, morphology can vary significantly from case to case. Granules can be fine or absent and the absolute number of lymphocytes can be in the normal range ***(70)***. In addition, other low-grade lymphoproliferative processes such as HCL can appear similar. Therefore, immunophenotypic analysis is vital for accurate diagnosis. FCM immunophenotyping reveals CD3+, CD7+/–, CD4–, CD8+, CD57+, and CD16 +/– [(results depend on antibody used with dim to negative staining with Leu 11 and positivity with VD2 or 8.28) and CD56- ***(70)***]. CD5 may be negative by immunohistochemistry ***(73)*** but is typically dim positive by flow cytometric analysis. Some T-cell LGL leukemias express the gamma delta T-cell receptor (TCR), whereas others express the alpha-beta TCR. Clonality of T-cell LGL proliferations can be determined by molecular studies or immunophenotyping with V alpha antibodies ***(74)***.

NK-cell LGL leukemias occur in a younger age group (median 39 yr old) and often present with systemic signs including fevers without infection, hepatosplenomegaly, anemia, thrombocytopenia, and lymphocytosis. Lymph node involvement is not uncommon. Morphology of the LGLs in the peripheral smear is similar to that observed in T-cell LGL. Immunophenotypically, the NK-cell LGL leukemias are typically CD3–, CD2+, CD5–, CD4–, CD8+/–, CD16 +/– (results depend upon antibody used), CD56+, and CD57– ***(1)***. NK-cell LGL leukemias do not have T-cell gene rearrangements and clonality is difficult to determine. NK-cell LGL leukemia is a more aggressive clinical course, whereas T-cell LGL is a more indolent process ***(75)***.

1.3.4. Immunophenotyping Other Selected Hematologic Disorders

1.3.4.1. Myelodysplastic Syndrome (MDS).

The myelodysplastic syndromes are a heterogeneous group of disorders characterized by peripheral cytopenias and multilineage dyspoiesis. The FAB classification is the most commonly used classification scheme and includes the following five categories: chronic myeloid monocytic leukemia (CMML), refractory anemia (RA), refractory anemia with ringed sideroblasts (RARS),

refractory anemia with excess blasts (RAEB), and refractory anemia with excess blasts in transformation (RAEBT) ***(76)***. Most patients present with a hypercellular bone marrow, although up to 25% have a hypocellular bone marrow ***(77)***. A significant number of patients with MDS are not easily diagnosed as having myelodysplasia based on bone marrow and peripheral blood findings ***(78,79)***. Although cytogenetic abnormalities associated with MDS have been described, they are found in only 20–60% of cases, depending on the laboratory performing the tests and the FAB classification ***(9)***. Investigators have begun to explore the utilization of flow cytometric immunophenotyping to improve diagnostic accuracy in this disease. Studies of the proliferative activity of myeloid precursors demonstrate a decrease in immature myeloid cell proliferation on progression within FAB groups ***(80)***. Bowen and Davis described abnormal patterns of expression of CD16 vs CD11b antigens by developing neutrophils in the bone marrow of patients with MDS ***(81)***. Both antigens normally increase during differentiation, with increases in CD11b being observed before upregulation of CD16. In 64% of the MDS patients studied, Bowen and Davis observed a greater fraction of granulocytes with low CD16 and CD11b expression, indicating delayed or abnormal maturation. Erythroid abnormalities were detected in MDS flow cytometrically based on decreased CD71 expression by erythroblasts. However, this was not disease specific, as decreased expression by erythroblasts was also observed in patients with anemia of chronic disease ***(82)***. As MDS is characterized by trilineage dyspoiesis, our group (M. Stetler-Stevenson) studied granulocytic, erythroid, and megakaryocytic precursors in a series of MDS and aplastic anemia bone marrows ***(83)***. We observed multiple granulocytic abnormalities in MDS and abnormal patterns of antigen expression in erythroid precursors. In addition, increased numbers of megakaryocytic cells were detected in bone marrow aspirates and correlated well with the presence of micromegakaryocytes. Although no single abnormality was diagnostic, demonstration of granulocytic, erythroid, and megakaryocytic abnormalities within a specimen was specific for MDS and more sensitive than morphology. These data indicate great promise for the utilization of FCM to improve diagnostic sensitivity in MDS.

1.3.4.2. Minimal Residual Disease (MRD)

Minimal residual disease detection, disease monitoring, and response to therapy all represent emerging areas for applying FCM. Two- and three-color FCM combined with molecular measurements can often determine the presence of the residual clone. The significance of such findings is sometimes unclear, particularly when present in asymptomatic individuals. However, when residual neoplastic cells are present and increasing in number during

monitoring, they often predict relapses weeks or months prior to morphological appearance of the clone. In our experience with the evaluation of CLL patients after treatment with fludarabine, we have not encountered a single complete remission using FCM evaluation (G. E. M., personal observation). Stewart and colleagues using a method of cluster analysis to normalize marrow subpopulations have shown that the flow cytometric relapse in AML can precede morphologic defined relapse by 6 mo. They are not able to predict relapse in patients whose leukemic cells are not found. whereas most are long-term relapses (>1 yr) about 10% can relapse in <1 yr (C. Stewart, personal communication).

Immune disease monitoring has also emerged as a new entity, particularly in the setting of bone marrow transplantation. As our understanding of hematopoietic engraftment has increased, investigators have begun to look at the kinetics of immune reconstitution following bone marrow transplantation. FCM will play a critical role in the analysis of acute and chronic GvHD and will undoubtedly continue to play a role in identifying the cell(s) responsible for graft vs leukemia (GVL).

1.3.4.3. Paroxysmal Nocturnal Hemoglobinuria (PNH)

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare, acquired clonal dysplastic disorder in which the patient's red blood cells exhibit excessive sensitivity to complement. Although the classic presentation is nocturnal hemolysis, often patient's present with pan cytopenia, or unexplained recurrent thrombosis. The defect represents a failure to properly synthesize the major anchoring glycophosphoprotein, phosphatidylinositol glycan (GPI). The sensitivity to complement is explained by the inability to anchor complement regulating proteins CD55 and CD59. The risk of thrombosis presumably relates to other GPI-linked proteins. The deficiency of CD59 in PNH can be demonstrated most clearly using FCM ***(40)***. There is some evidence that CD66 may be more sensitive than CD59 in detecting deficient GPI-anchored proteins. The use of FCM has significant advantages over the previous use of the cumbersome Ham's test for the laboratory evaluation of PNH and in many cases has been replaced by FCM. In PNH, the loss of GPI-linked markers is more often seen in red blood cells and granulocytes, compared to monocytes, and least often in lymphocytes. Because lymphocytes are seldom affected, it is prudent to evaluate red cells and granulocytes. Presently there is no clear information about platelets. It may be possible to pick up the defect in marrow cells before it is manifested in the blood. It also appears that the defect can be manifested as an incomplete loss of a marker, i.e. the cells may show decreased mean fluorescence on the entire population, there may be a subset with decreased or absent expression, or there may be absent expression on the entire population.

GPI-linked markers of PNH that have been used include CD59, CD48, CD58, CD67 (now CD66b), CD55, CD16, and CD14. It is important to combine several reagents because of variable changes in the different PI-linked markers and the variability between cell lineages. Finally, the expression of the defect can change over time in terms of affected lineages (***85–89***; Ken Ault, personal communication, Cytometry List, May 1998).

1.3.4.4. CD34 Analysis

Early functional studies of bone marrow engraftment involved colony-forming unit (CFU) assays. CFU assays require weeks for completion, are labor intensive, and show considerable interlaboratory variation. With the discovery of CD34 and the use of mobilized peripheral blood stem cells (PBSC), a flow cytometric, and ultimately an image-based assay, emerged. The initial FCM studies of CD 34 cells showed an interlaboratory variation of over 1000% in this country and in Europe ***(17,18)***. We suggested that QFCM might be useful in reducing interlaboratory variation. While this interlaboratory variation was being examined, another area emerged in the setting of CD34 determinations involving CD34 subsets. It became obvious that the type of CD34 cells involved in determining outcome were important. Serke has noted that the number of transplanted CD34-expressing cells coexpressing the CD41 antigen correlate with platelet recovery following high-dose therapy and transplantation ***(44)***. A dose response between the number of CD34 cells per kilogram and the time required for engraftment has been suggested ***(90)***. The emergence of ex vivo expansion of peripheral blood and cord blood progenitor and stem cells for clinical use will undoubtedly continue to make use of stem subset analysis. Finally, the emergence of tumor vaccines using in vitro immunization of dendritic cells represents a parallel opportunity for the flow cytometric analysis of monocyte or marrow dendritic cell markers associated with cell derivation and maturation. The role of flow cytometric sorting of antigen-specific, cytotoxic T cells and their ex vivo expansion may also be a further development in the clinical use of flow sorted cells. In this regard, multiparameter FCM will be very useful to identify the presence of antigen-specific cytotoxic T cells.

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10

Flow Cytometric Analysis of Hematologic Neoplasia

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1. Introduction

Flow cytometry (FCM) is a powerful technology that allows the rapid analysis of cellular components such as surface and intracellular antigens, or DNA content. The measurements are fast and are based on optical signals emitted by cells labeled with fluorochromes as they flow suspended in a liquid medium through an intense laser beam. The signals include scattered light, which provides information on cell size and granularity, and fluorescence derived from dyes or fluorochrome-labeled antibodies bound to specific cell components.

Leukemias and lymphomas have been traditionally diagnosed and classified by microscopic means using smears or tissue sections colored with conventional stains. These stains have been useful in the visualization of cells or tissue structures, but they lack the specificity required to recognize cell of origin, differentiation stage, or growth behavior. Monoclonal antibodies have added specificity and biological significance, and FCM contributed objectivity and quantitation to the observations. These technologies have revolutionized the manner in which we diagnose and characterize hematologic neoplasia.

FCM has been used in the study of human lymphoma and leukemia since the late 1970s. Initially, most studies were focused on the analysis of DNA. Subsequently, as monoclonal antibodies became available, the attention shifted to the analysis of cellular antigens. Although DNA content analysis provided prognostic information and an assessment of proliferative capacity ***(1–3)***, the studies of cellular antigens permitted the identification of cell of origin, a clear delineation of cell differentiation and the recognition of abnormal phenotypes ***(4–7)***. The latter is used extensively in the diagnosis and classification of hemopoietic neoplasia and in the detection of minimal disease ***(8–15)***.

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2. Materials

2.1. Instruments and Equipment

1. Flow cytometer.
2. Biological hood.
3. Refrigerated centrifuge.
4. Ice bath.
5. Vacuum apparatus.
6. Vortex.
7. 37°C water bath.
8. Scalpels.
9. Forceps.
10. Wire mesh screen (and wire mesh pestle—optional) #80 mesh = (80/in.).

2.2. Plastic Ware

1. Disposable Petri dishes.
2. Disposable transfer pipets.
3. 50 mL conical centrifuge tubes.
4. 15 mL conical centrifuge tubes.
5. 12×75 mm polystyrene test tubes.
6. Precision adjustable pipettors.
7. Disposable pipet tips.

2.3. Reagents

1. Phosphate-buffered saline with 0.1% NaN_3 (PBS).
2. Lysing buffer: 8.29 g NH_4CL, 1.0 g $KHCO_3$, 37 mg EDTA, 1 L distilled water.
3. Cell preservation media (CPM) containing media such as RPMI or Hank's solutions with 10% fetal or newborn calf serum and a mixture of antibiotics.
4. Fetal or newborn calf serum.
5. Monoclonal antibodies—used as recommended by manufacturer.
6. 100% ethanol chilled in ice bath.
7. 0.5% paraformaldehyde.
8. Cell permeabilization solution—used as recommended by manufacturer.
9. PI (propidium iodide: 50 mg/mL in 1.12% sodium citrate.
10. RNase.

3. Methods

3.1. Sample Preparation

FCM analysis of hemopoietic neoplasia can be performed in a number of specimen types, but the analysis requires the samples to be monodispersed in single-cell suspensions. Samples are most commonly bone marrow aspirates, peripheral blood, and lymphoid tissues but also include body cavity fluids and tissues like skin, mucosa, and solid organs (e.g., liver, brain, soft tissue) (*see*

Note 1). Each sample type may require a special handling procedure to obtain cell suspensions. Blood, bone marrow, and other fluids are the easiest to prepare, as they already contain single cells in suspension. The cells in these samples can be directly stained with antibodies prior or following erythrocyte lysing (*see* **Subheading 3.1.2.**). In contrast, solid tissues require cell disaggregation to obtain single cells for analysis, a procedure that is accomplished by mincing the tissues in a saline solution or tissue media, forcing the fragments through needles with syringes or gently rubbing them onto a metal mesh (*see* **Notes 2** and **3**). Mechanical devices created for this purpose may also be used ***(16)*** including the commercially available Medimachine (Dako Corp., Carpinteria, CA, or BD Biosciences, San Jose, CA). It is always a good practice to obtain morphological controls of the suspensions obtained by performing smears or cytocentrifuge preparations that are stained for microscopic observations (*see* **Note 2**).

3.1.1. Obtaining Single-Cell Suspensions from Tissues

1. Perform work in a biological hood.
2. Perform gentle-touch imprints, preferably on a freshly cut tissue surface.
3. Pour RPMI or Hank's solutions into a Petri dish. Immerse screen in fluid.
4. Using forceps place the tissue on top of the screen. Be certain that liquid covers the screen.
5. Gently use scalpels to mince the tissue with quick straight strokes.
6. Using a disposable transfer pipet, aspirate cells and media in the Petri dish and eject through the screen to enhance cell separation.
7. Collect the cell suspension in the Petri dish with a transfer pipet and place in an appropriately labeled conical tube. Add twice the volume of media.
8. Proceed to erythrocyte lysing, in **Subheading 3.1.2.**
9. Dispose of scalpels in an appropriate biohazard box.
10. Place any remaining tissue fragments back in the original container and dispose of container according to facility policy.
11. Decontaminate and wash screen with appropriate solution.
12. Clean work surfaces of hood with appropriate solution and turn on UV light for 15 min to decontaminate work surface.

3.1.2. Erythrocyte Elimination

If abundant red cells are present in a cell suspension, they need to be excluded from the analysis, either by density gradient or lysis. Density gradient centrifugation ***(17,18)*** can be used prior to cell staining to eliminate red cells, granulocytes, and nonviable cells from cell suspensions. Currently, the use of gradient centrifugation is discouraged, as this procedure can lead to selected cell loss, including the cells of interest ***(19,20)***. Most laboratories eliminate nonnucleated red cells from the suspension by using red-cell lysing

solutions. This lysing may be performed prior to or following antibody staining. The first approach is used when circulating antigen may block the binding of antibodies, typically in the analysis of cell surface expression of immunoglobulins. Granulocytes and dead cells are excluded from the analysis on the basis of light scatter ***(21)*** and fluorescence signals from DNA-binding fluorochromes that penetrate nonviable cells ***(22)***, respectively. The following is a method for erythrocyte lysing prior to cell surface staining:

1. Keep sample at room temperature.
2. Place 4–5 mL of blood, bone marrow, or cell suspension into an appropriately labeled 50 mL conical tube.
3. Add 45 mL of lysing buffer to the 50-mL conical tube. Invert occasionally. Keep at room temperature for 10 min. If more than 5 mL of blood or bone marrow is to be lysed, use additional 50 mL tubes.
4. Centrifuge at 400*g* for 5 min at room temperature. Aspirate supernatant and gently mix pellet to resuspend. Add 40 mL phosphate-buffered saline (PBS) and repeat this procedure twice.
5. Resuspend cells in CPM and adjust to desired concentration.

3.2. Antibodies and Fluorochrome Selection

The analysis of hematologic samples usually requires numerous antibodies. Cells from each sample are distributed among several tubes (or wells in a microtiter plate), each containing usually two to four antibody combinations designed to be especially pertinent to the type of sample, the presumptive diagnosis, or the concurrent morphologic findings in that case. At present, there is no standardization in the selection of antibody and fluorochrome combinations applied to hematologic malignancies. The choice of antibodies is variable among laboratories and depends on factors such as the type of laboratory involved in the analysis, the personal preferences of the laboratory professional, or cost considerations. According to recent surveys, laboratories apply an average of 15–20 reagents per case ***(23)***, although it is currently recommended that a higher number of antibodies be used.

The assembly and validation of appropriate reagent combinations is ultimately the responsibility of the laboratory performing the analysis. Each laboratory should be acquainted with the particular binding and fluorescence properties of the reagents it uses. It is important to assess the results of the analysis in normal samples before determining if abnormalities are present in clinical samples. Because most clinical samples contain normal cells, the laboratory may also use these normal elements as routine validation targets ***(24)***.

It is beyond the scope of this publication to advocate any particular set of antibody combinations for the detection and characterization of hematologic neoplasia, and the reader is referred to a number of recent publications regard-

ing this issue ***(25,26)***. In general terms, it is recommended that antibody panels used in the analysis of hematologic neoplasias be sufficiently ample as to allow the identification of all normal and potentially abnormal elements in the various tissues studied. A restricted number of reagents may limit the ability to recognize neoplastic cells that express antigens in an aberrant manner, or low-level tumor involvement.

3.3. Antigen Staining

3.3.1. Surface Antigen Staining

Cell antigens can be detected on the cell surface or intracellularly. Cell surface antigen staining is performed on viable cells, which can be stained with single or multiple antibodies labeled with appropriate fluorochromes. Multicolor immunofluorescence is increasingly used in FCM since this approach has notable advantages over single-reagent staining, particularly in the recognition of cellular heterogeneity and in samples with a limited number of cells. The use of multiple antibody and fluorochrome combinations, however, poses potential problems such as spectral overlap, reagent interaction, or antigen blocking, which require performing appropriate quality control and validation procedures ***(24)***. Cell surface antigen staining in erythrocyte-depleted cell suspensions can be performed using one of the following two methods:

3.3.1.1. Standard Tube Method

1. Label 12 × 75 test tubes with name, date, and antibody.
2. If cell suspension is not in CPM, coat tubes with fetal or newborn calf serum.
3. To each tube, add approximately 10^6 cells.
4. Bring total volume in each tube to 2 mL with PBS.
5. Centrifuge tubes at 4°C for 5 min at 400*g* to pellet cells.
6. Aspirate supernatant using vacuum apparatus.
7. Gently vortex cell pellet.
8. Resuspend cell pellet by adding 250 µL PBS.
9. Add manufacture's suggested volume of conjugated monoclonal antibody to the cell suspension (*see* **Note 4**).
10. Vortex. Incubate cells in the dark on ice for 15 min.
11. Add 2 mL cold PBS to each tube.
12. Centrifuge tube at 4°C for 5 min at 400*g*. Aspirate supernatant.
13. Repeat **steps 11** and **12**.
14. After second wash, resuspend cells in 500 µL of cold PBS. Keep the cell suspension in the dark on ice until it's ready to be analyzed (*see* **Note 5**).

3.3.1.2. Microtiter Plate Method

1. Coat the wells of a Falcon 96-well U-bottom assay plate (Becton Dickinson) with 1% bovine serum albumin.

2. Dispense antibody and cells in reduced amounts, but in equal proportions as the tube method (*see* **Subheading 3.3.1., step 1**), to the wells of the assay plate.
3. Incubate cells in the dark on ice for 15 min.
4. Centrifuge plates at 4°C for 5 min at 400*g* to pellet cells.
5. Discard the supernate by quickly inverting plate (flicking) over a biological waste container.
6. Add 200 µL of cold PBS to wells. Tap the sides of the assay plate gently to resuspend the cell pellet.
7. Centrifuge plates at 4°C for 5 min at 400*g* to pellet cells.
8. Repeat **steps 6** and **7**.
9. Resuspend cells in 200 µL of cold PBS (or less, depending on cell number). Transfer the cells to tubes and keep them in the dark on ice until they are ready to be analyzed.

3.3.2. Intracellular Antigen Staining

For detection of intracellular antigens, the cells are fixed to maintain structural integrity, and permeabilized to allow antibodies to reach the appropriate intracellular targets ***(27–29)***. Intracellular antigen staining is a more laborious procedure than cell surface antigen staining, but is gaining increasing importance, as many products that play key roles in hematological malignancies are located in the cytoplasm or the nuclei. Often intracellular antigens are detected in conjunction with cell surface antigens ***(30)***. This combined procedure provides excellent data, but each combination may require optimization of the staining conditions, as fixation and permeabilization could affect the stability of the antigen–antibody complexes on the cell surface or the intracellular antigens may be lost during or subsequent to permeabilization.

1. If intracellular staining is performed in conjunction with surface antigen staining, follow the aforementioned surface-staining procedure through **step 13** in **Subheading 3.3.1.1.** first.
2. To the cell pellet add 500 µL of 0.5% paraformaldehyde, vortex gently, and incubate on ice for 15 min.
3. Add 2 mL of PBS to each tube and centrifuge at 4°C for 5 min at 400*g* to pellet cells. Aspirate supernatant. Repeat this step twice.
4. Add manufacturer's suggested volume of a permeabilization solution to the cell pellet and incubate in the dark on ice for 15 min (*see* **Note 6**).
5. Repeat **step 3**.
6. Antibody staining as per surface-staining procedure above (start at **step 9** in **Subheading 3.3.1.1.**) but increase all incubation times from 15–30 min.

3.4. DNA Staining

Cellular DNA content analysis provides information in DNA ploidy and cell cycle phases. Ploidy is used mainly as a prognostic indicator in pediatric

acute lymphoblastic leukemia ***(2,31)***, and cell cycle phase analysis provides information on cell growth and is useful in grading lymphomas ***(1)***. In most cases, DNA is stained with propidium iodide (PI), an intercalator that binds to double-stranded nucleic acids. PI staining requires cell permeabilization, which is accomplished by either fixation or detergents. Cellular DNA can be analyzed by itself or in combination with fluorescein isothiocyanate (FITC)-labeled antibodies, as PI fluorescence emission can be easily separated from the green fluorescence signals of FITC ***(32–35)*** (*see* **Note 7**).

1. Label one 12 × 75 test tube for each patient.
2. If cell suspension is not in CPM, coat tubes with fetal or newborn calf serum. This will ensure proper cell pelleting.
3. To each tube add approximately 10^6 cells (*see* **Note 8**).
4. Bring total volume in each tube to 2 mL with PBS.
5. Centrifuge tubes at 4°C for 5 min at 400*g* to pellet cells.
6. Using vacuum apparatus aspirate supernatant.
7. Gently vortex cell pellet.
8. Resuspend pellet by adding 250 µL of PBS to cell pellet.
9. Cool the cell suspension in the dark on ice for 5 minutes.
10. Gently mix 500 µL of chilled 100% ethanol to the tubes containing the cooled cell suspension (*see* **Note 9**).
11. Incubate in the dark on ice for 15 min.
12. After incubation, add 2 mL of cold PBS to each tube, centrifuge at 4°C for 5 min at 400*g*.
13. Aspirate supernatant. Repeat **steps 12** and **13**.
14. Add 500 µL RNase to each tube and incubate in 37°C water bath for 15 min (*see* **Note 10**).
15. Add 500 µL of PI solution to each tube and incubate at room temperature for 15 min.
16. After the 15-min incubation, the tubes are ready to run on the flow cytometer.

3.5. Combined Antigen and DNA Staining

1. Label one 12 × 75 test tube for each patient.
2. If cell suspension is not in CPM, coat tubes with fetal or newborn calf serum. This will ensure proper cell pelleting.
3. To each tube add approximately 10^6 cells.
4. Bring total volume in each tube to 2 mL with PBS.
5. Centrifuge tubes at 4°C for 5 min at 400*g* to pellet cells.
6. Using vacuum apparatus, aspirate supernatant.
7. Gently vortex cell pellet.
8. Resuspend pellet by adding 250 µL of PBS to cell pellet.
9. Add the manufacturer's recommended volume of FITC-conjugated antibody to each tube containing 10^6 cells.
10. Vortex gently. Incubate tubes in the dark on ice for 15 min.
11. After incubation add 2 mL cold PBS to each tube.

12. Centrifuge tube at 4°C for 5 min at 400*g*.
13. Repeat **steps 11** and **12** twice.
14. Add 500 µL of 0.5% paraformaldehyde to each tube (*see* **Note 11**).
15. Incubate for 15 min in the dark on ice.
16. After incubation, add 2 mL of cold PBS to each tube, centrifuge at 4°C for 5 min at 400*g*.
17. Aspirate supernatant. Repeat **steps 16** and **17**.
18. Add 500 µL RNase to each tube, cap, and incubate in 37°C water bath for 15 min.
19. Add 500 µL of PI solution to each tube and incubate at room temperature for 15 min.

3.6. Data Analysis

3.6.1. Immunophenotype Analysis

There are no established criteria for how flow cytometric data should be analyzed in hematologic neoplasia. Traditionally, the immunophenotypic data analysis of lymphoma and leukemia has been based on a similar approach as the one used for quantitation of lymphocyte subsets. Thus, many laboratories apply a strategy that consists of initially selecting or "gating" a population of interest (e.g., "lymphocytes"), followed by a calculation of percent of cells positive for each antigen studied within this population. However, the gating strategy based on forward versus right-angle light scatter signals, and discrimination of positive from negative cells, extensively used in the analysis of T-cell lymphocyte fractions, cannot always be accurately applied to hematological neoplasia where the cells do not resemble normal elements. Therefore, the results in these cases are often imprecise ***(36)***. Furthermore, sampling variation may also affect numerical values.

We recommend an approach to data analysis that is similar to that used in the observation of microscopic slides. The analysis of light scatter and immunofluorescence distributions from normal cells exposed to antibodies results in patterns of graphical data that are characteristic for each tissue analyzed, reflecting the various cell lineage and differentiation stages. As in microscopic preparations the presence of neoplastic cells distorts these normal patterns. Thus, a visual inspection of the graphical data, usually aided by gating of cell populations of interest, permits the identification and characterization of abnormal (neoplastic) cells ***(36)***. The advantage of this approach is that one can immediately detect the presence of even low numbers of neoplastic cells that may not necessarily be reflected in abnormal numerical results. Another benefit is being able to recognize changes in fluorescent intensity, a component of the analysis that is important in the interpretation of the data and the precise characterization of many neoplasias. This visual approach for data

analysis does not depend on numerical data but it should not preclude the quantitation of defined cell subpopulations, if applicable or desirable.

Data analysis techniques may also vary depending on the sample, the nature of the clinical problem and availability of other pertinent information. Thus, the approach used in the analysis of lymphoid tissues may be different from that used in bone marrow or blood. The strategy may also be different if the sample is post-therapeutic and previous data on the neoplastic cells are available. Gating may vary with diseases and samples. Thus, the gating for acute leukemia is not the same as that used in the recognition of lymphoproliferative disorders. CD45 immunofluorescence intensity, in conjunction with side-light scatter signals, is often used in the recognition of various cell subpopulations in blood and bone marrow, including leukemic blasts ***(37–41)***, which can be gated and analyzed independently based on other antibodies. A similar approach can be used in B-cell chronic lymphoproliferative disorders or lymphomas using B-cell markers, such as CD19 or CD20, or T-cell neoplasias using pan-T cell markers. Similarly, forward light scatter properties reflecting cell size, can be utilized in the gating of large-cell lymphomas.

3.6.2. DNA Analysis

With regard to DNA content analysis ***(42)***, ploidy changes are measured by the DNA index (DI), which is the ratio between the fluorescence of the G1 peak of the neoplastic cells and that of the G1 peak of the standard diploid control (e.g., normal lymphocytes). This measurement could be obtained by using commercially available computer programs, which also analyze cell cycle fractions. The proper assessment of cell cycle fractions in hematologic neoplasia, however, requires an understanding of the cellular composition of the samples. Very often, involved lymph nodes, bone marrow, or other tissues contain a considerable number of normal cells admixed with the neoplastic elements that may even outnumber tumor cells. Unless corrected for the presence of these normal cells, the analysis of "neoplastic" cell cycle fractions may be incorrect if a DNA content distribution of normal and neoplastic cells overlap extensively. Thus, if the neoplastic cells are diploid or close to diploid and an unknown number of nonneoplastic cells are present in the preparation, an accurate calculation of the neoplastic cell cycle fractions using only DNA content analysis is impossible. In these cases, a marker for either the neoplastic or the normal cells should be simultaneously used with DNA staining for a more correct analysis of cell cycle kinetics of the neoplastic cells ***(43)***. As with microscopic interpretation, the laboratory professional involved in data analysis should be cognizant of the clinical, morphological, and other pertinent laboratory information related to the case being analyzed, so that an appropriate report can be generated and communicated to the treating physician.

4. Notes

1. Specimens should be submitted for analysis as soon as they are obtained. Solid tissues should be maintained in a moist environment to prevent dehydration. This could be accomplished by wrapping the tissues in saline or culture media-soaked gauze, or similar material. In practice, it is known that most samples would tolerate transportation for approximately 24 h and many would withstand 48–72 h before critical changes occur. Cell viability may deteriorate with time, but this process varies greatly depending on the type of cells in the sample. Freezing cells is an alternative procedure if the analysis is delayed. However, most clinical laboratories lack the necessary facilities for cell freezing. There is no consensus on the best temperature to transport specimens, although it is likely that lower temperatures (approx 4°C) are better to maintain cell viability.
2. Cell suspensions obtained from solid lymphoid tissues do not always contain the neoplastic cells of interest. Tumor cells may be trapped in a meshwork of fibers, which would prevent cell release into suspension. Alternatively, the tumor cells may be fragile and unable to withstand the harsh procedures involved in cell disaggregation, washing, staining, etc. Another consideration is that lymphoid tissue may contain fibrous material or may be necrotic so that the resulting cell suspension may not necessarily contain the tumor of interest. For these reasons, cytospins are prepared to insure that the cells of interest are present in the suspension. These preparations are compared with frozen sections, touch imprints or smears from the original samples for quality control.
3. Cell disaggregation procedures can also be applied to bone marrow biopsies and are useful in instances in which the bone marrow aspirates do not yield sufficient numbers of cells (dry tap).
4. The procedure described is for single antibody staining. Generally, preparations include two or more antibody combinations per tube in which case the antibodies are added simultaneously. It is advisable to check antibody titers and specificity using appropriate cell targets.
5. Non-viable cells can pose problems of nonspecific antibody binding when cell surface antigens are analyzed. Dead cells may be excluded from the analysis by the simultaneous use of propidium iodide (PI) or similar DNA-binding dyes, which do not penetrate cells with intact membranes. This approach, of course, is not valid when analyzing intracellular antigens, as the latter requires cell membrane permeabilization.
6. There are several kits on the market that have both a fixative and a permeablizing reagent. If using one of these kits follow manufacturers' suggested procedure for volumes and incubation times.
7. Nuclear DNA can be analyzed in formalin-fixed, paraffin-embedded tissues used in histologic preparations. This allows retrospective studies, but the technique tends to result in poorer quality analysis and does not permit simultaneous staining of DNA and antigens.

8. For quality control and ploidy measurements, normal human lymphocytes are commonly used as either external or internal standards mixed with the cells in the samples prior to staining.
9. Other fixatives may be used, but ethanol preserves cell integrity while allowing excellent quality of DNA staining.
10. RNase is required, as PI binds to all double stranded nucleic acids.
11. Ethanol may be an alternative fixative. Although paraformaldehyde may cause DNA crosslinking and higher coefficient of variations in the DNA measurements, it seems better for preserving cell surface antigen staining.

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11

In Situ Hybridization in Flow Cytometry

Victoria L. Mosiman and Charles L. Goolsby

1. Introduction

Molecular approaches to diagnostic questions in clinical medicine are greatly impacting the way researchers and clinicians investigate and treat disease. By combining molecular techniques with classical immunologic tools such as flow cytometry (FCM; ***1–3***), one can begin to more fully understand and appreciate the role of cellular heterogeneity in disease processes. The marriage of these two powerful techniques, termed molecular cytometry, will, in one instance, allow investigators to explore expression of nucleic acid sequences in subpopulations of cells defined by immunologic phenotype while, conversely, making it possible to examine the heterogeneity of cellular characteristics within populations identified by the presence of specific nucleic acid sequences or gene expression. Future developments may result in several advantages for the patient that may include, but are not limited to, earlier detection of viral infection, earlier and more sensitive detection of malignancy, and higher sensitivity and resolution of small populations of infected or aberrant cells. These developments may also assist in the identification of therapeutically resistant populations within a neoplasia, more effective and specific monitoring of therapy, and possibly the identification of new and disease-specific targeted therapies based on genetic information. The characterization and assessment of cellular heterogeneity is clearly key to understanding disease onset, progression, and therapeutic response in both infectious disease and in human malignancies.

Although the ability to detect a low number of copies, or single copy, of a DNA or RNA sequence *in situ* by FCM is still evolving, many groups have already had success in proving the potential for *in situ* techniques in the detec-

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tion of relatively high copy number (greater than several hundred copies) DNA ***(4–7)*** and RNA ***(8–16)*** targets. In addition, detection of low copy number targets has been reported ***(17,18,22)***. Typically, one of two general approaches have been taken in approaching fluorescent *in situ* hybridization (FISH) detection of specific nucleic acid sequences in FCM. In one case, target sequences are first amplified to a high copy number followed by FISH detection of that amplified product, or in the second, one performs a FISH procedure without preamplification of target sequences. **Figures 1** and **2** demonstrate general schematics or overviews of these two basic schemes. Essentially, amplification approaches revolve around attaining levels of fluorescent intensity in positive cell populations that are readily distinguishable above background levels of nonspecific binding and autofluorescence. For a very high number of copies of a specific target sequence, one may be able to detect positive cells employing a limited number of labeled probes in a direct FISH assay. However, for most applications detecting lower numbers of copies (< 1000 copies per cell) some amplification of the signal must be done. Typically, one can either amplify the target sequence of interest ***(17–21)***, or amplify the detected signal of the probe(s) specifically directed to that target ***(22,23)***. Target amplification, typically performed prior to hybridization, includes techniques such as *in situ* PCR ***(17,18)*** and *in situ* reverse transcriptase-polymerase chain reaction (RT-PCR) ***(17,43)*** amplification with slight modifications for use with cells in suspension. In this scenario, primers are designed to flank a known sequence of interest to generate hundreds of thousands or even millions of copies of the target sequence *in situ*. This amplified target is then detected using one or limited number of labeled probes specific for that amplified sequence employing a FISH technique. Techniques employing a fluorescein-labeled primer have also been reported ***(20)***, although a detection sensitivity of only approximately 1000 copies per cell was seen. We have found the former approach of amplification coupled with FISH detection of the amplified product to be more sensitive in the detection of very low copy number sequences. Conversely, in the alternative general approach, signal amplification employs the use of either multiple oligonucleotide probes, which in aggregate span or hybridize to all or major portions of a target sequence of interest ***(16,24)***, or amplification of the signal from a small number of labeled probes ***(25)***. A number of authors have also employed probes generated by labeling a construct containing a homologous portion of a target sequence of interest to provide probes that span a large proportion of a sequence of interest ***(6,7,13)***, however, at this point, only relatively high copy number targets have been reported to be detected utilizing this approach.

Both types of approaches have been successfully demonstrated in the field of virology ***(23,26,28–31)***, specifically the study of human immunodeficiency virus (HIV) infection ***(17,18,22,24)***, as well as in areas related to hematologic

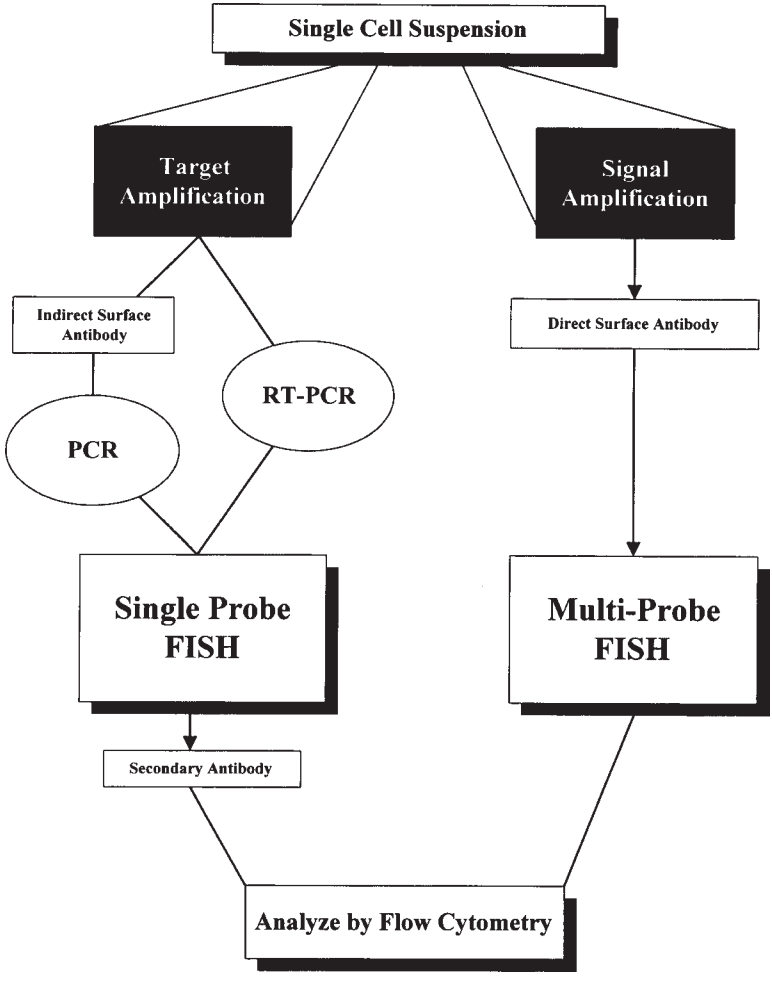

Fig. 1. Schematic of alternative approaches to *in situ* detection of cellular nucleic acid targets.

malignancy ***(35,36,43,47)***, immunology ***(12–14)*** and a variety of other applications ***(4–11,15,16,19–21)***. Use of *in situ* RT-PCR or *in situ* PCR combined with immunophenotype has aided in determining whether cellular populations are latently or actively infected ***(17)***, and whether other cellular features have been altered by viral gene expression ***(18)***. Direct hybridization techniques have also been used to identify cellular subpopulations that are potentially supporting active replication of virus ***(22)***. Although it remains to be definitively proven, enticing data has been reported suggesting that "in cell" or *in situ* measures of HIV viral load, as opposed to plasma viral load determinations, add

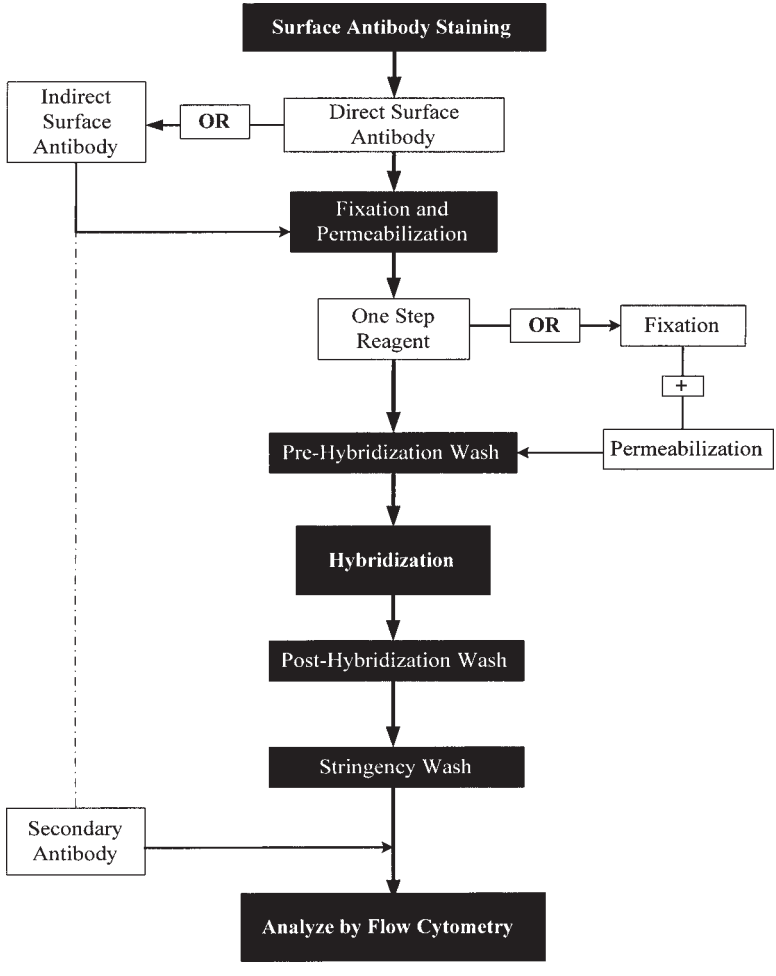

Fig. 2. Schematic representation of FISH protocol with reagent options.

additional information in terms of HIV disease progression and therapeutic response ***(22)***. Similar potential exists for direct *in situ* detection of other viruses such as cytomegalovirus (CMV), human papilloma virus (HPV), and Epstein-Barr virus (EBV) ***(26–30)***. The ability to detect EBV in cell lines, coupled with simultaneous analysis of cell surface markers has been shown ***(31)***, and preliminary results in patient samples look promising ***(23)***. Monitoring EBV and CMV may prove to be particularly fruitful in the area of transplant medicine where sensitive and rapid detection of viral activation is important in both donor and recipient populations ***(32–34)***.

In addition to virology, several other areas of transplant medicine are potential areas for the application of molecular cytometry methods. A few examples include documentation of successful allogeneic engraftment and detection of minimum residual disease cells ***(35)***. Monitoring of sex-mismatched bone marrow transplants for chimerism within distinct cell lineage has shown good correlation to standard techniques ***(36)***. This procedure not only reduces several time consuming steps such as sorting of specific cell fractions, and subsequent PCR amplification, but may prove to be more quantitative than standard PCR for posttransplant monitoring ***(37–39)***. Other applications include detection of chimerism based on cellular detection of major histocompatibility complex (MHC) regulation following allogeneic engraftment ***(40)***. This approach, which successfully coupled cellular immunophenotype with *in situ* PCR amplification of donor-vs.-recipient histocompatibility genes, enabled researchers to examine differences in cellular and humoral immune response after kidney transplants.

The extension of molecular techniques to FCM *in situ* detection of cytogenetic abnormalities associated with ***(41)***, or in some cases defining ***(42)*** specific hematologic malignancies has only begun. FCM-based *in situ* RT-PCR approaches have been used to detect cytogenetic abnormalities at the cellular level. An example of this includes amplification of the *brc:abl* fusion transcript in chronic myeloid leukemia ***(43)***. Direct FISH techniques were utilized to probe for sequences in the centromeric region of chromosome 17 in non-small-cell lung carcinoma, showing good correlation of chromosome copy number as measured by relative fluorescent intensities in FCM and fluorescence microscopy ***(46)***. Additionally, direct FISH detection of patient-specific immunoglobulin heavy-chain variable regions in multiple myeloma has been used to enrich malignant cells ***(47)***. However, the real potential of these approaches in FCM has not been realized at this point, as no reports of successful *in situ* RT-PCR detection coupled with other immunofluorescence-based measurements have been reported in cytogenetic applications.

Significant technical developments remain to be made before the potential power and impact of these molecular cytometry approaches will be realized. Nonetheless, these advancements clearly will permit sophisticated, multiparametric studies of the cellular heterogeneity present in most diseases. It appears that soon most, if not all, hematopoietic malignancies will be defined by their cytogenetic abnormality. The classic examples of t(9;22) in chronic myelogenous leukemia ***(48–50)*** and t(15;17) in acute promyelocytic leukemia ***(51,52)*** have now been joined by a fairly lengthy list of specific cytogenetic abnormalities in other malignancies that are too numerous to list in their entirety, but a brief synopsis is included in **Table 1**.

Table 1
A Partial List of Cytogenetic Abnormalities in Hematopoietic Malignancies: Potential *in situ* Cellular Targets

Malignancy	Abnormality	Reference(s)
CML	t(9:22)	***42,48–50***
AML M2	t(8;21)	***53,54***
AML M3	t(15:17)	***41,52***
AML M4	inv(16)	***63***
Childhood B-ALL	t(12;21)	***55,56***
B-ALL	t(8;14)	***57,58***
Therapy-related ALL	t(4;11)	***64***
	t(9;11) (and other 11q23 abnormalities)	***64***
Burkitt's lymphoma	t(8;14)	***57,58***
Follicular lymphoma	t(14;18)	***59,60***

This is but a partial list of potentially interesting targets for the detection approaches described in this report. It should be noted however, that although cytogenetic markers assist in classification of disease, they also can carry significant diagnostic and prognostic information. Interestingly, with respect to the latter, several acute leukemias are characterized by abnormalities that involve rearrangements of the genes coding for the α- and β-chains of the core binding transcription factor (t(8;21), t(12;21), and inv (16) from **Table 1**). These examples define a heterogeneous group of malignancies that may account for as much as 15–20% of acute leukemias, all of which have a relatively good prognosis. Conversely, other chromosome translocations carry a poor prognosis such as the t(1;19). It is obvious that facilitating rapid detection of these cytogenetic abnormalities as part of routine immunophenoptypic workups for leukemias and lymphomas would be of significant advantage.

Perhaps more importantly, however, would be utilization in the definitive detection of minimum residual disease cells for therapeutic monitoring. This would not only allow for characterization of residual cells, but also potentially permit simultaneous monitoring of that cellular population's response to therapy. In many residual disease settings, simply knowing that a patient is positive for a given translocation at the PCR or RT-PCR level is not adequate. For example, in CML, significant numbers of patients in long-term clinical remission remain RT-PCR positive for the *bcr:abl* fusion transcript ***(42,44)***. It has been postulated that possibly it is the number ***(3)*** of aberrant cells that is more important in terms of prognosis rather than their mere presence. Cellular molecular cytometry techniques will provide the tools necessary to assess questions of cell number while simultaneously determining other characteristics of

residual disease cells. Some interesting examples include properties such as proliferation status, drug resistance phenotype, or expression of other prognostically relevant markers. These advancements will facilitate a more complete understanding of the biology of minimum residual disease, and hopefully, permit determination of patients who are going to clinically recur, and thus may need more intensive therapy vs. those that are not.

Furthermore, in most instances, translocations point to key altered proteins, which are probably important in terms of the cause and characteristics of each malignancy. As such, they may serve as ideal targets for the development of new therapeutics. Thereby reinforcing the need for the continued development of molecular cytometry approaches to detect these abnormalities and their associated protein products at the cellular level. Finally, the interaction of these altered proteins with regulatory proteins of multiple, and in some instances redundant, pathways that control cellular proliferation, apoptosis, differentiation, and resistance will be paramount to understanding therapeutic response as well as disease progression. Thus, an exciting future is upon us as we become better able to implement more sophisticated multiparametric molecular cytometry techniques.

In the remainder of this chapter, we present some basic principles that underlie the design of protocols and reagents utilized for the detection of specific nucleic acid sequences within cells coupled with ability to measure other cellular parameters such as surface immunophenotype. Because most of the protocols described have arisen from analysis of viral sequences in lymphocytes, some modification may be necessary for the reader's specific application. Still, this should serve as a framework from which to begin, and we have tried to indicate areas where modifications may be needed for other specific applications.

2. Materials

2.1. General Supplies

1. 1X phosphate-buffered saline (PBS) calcium chloride, magnesium chloride, and sodium azide free.
2. Diethyl pyrocarbonate (DEPC) treated dH_2O.
3. 20X standard saline citrate (SSC) buffer (3 *M* NaCl, 0.3 *M* sodium citrate) diluted to working concentration.
4. Glass-distilled molecular-grade formamide.
5. Triton X-100.
6. Saponin.
7. Single-stranded sheared salmon sperm DNA.
8. Direct labeled probe or probes specific for target of interest (*see* **Subheadings 3.6.2.** and **3.6.2.1.**, and **Note 1**).
9. Disposable 12 × 75 mm Falcon tubes (polypropylene or polystyrene).

10. 15 mL Falcon tubes (polypropylene or polystyrene).
11. Pipetman or equivalent pipettors (1–20 µL, 20–200 µL, 200–1000 µL).
12. Pipets (1–10 mL).
13. Vortex mixer.
14. Centrifuge.
15. Vacuum aspirator.
16. Water bath or incubator.
17. Disposable gloves.
18. Differential cell counter enumerating white blood cells (WBCs) or hemocytometer.
19. Flow cytometer.

2.2. Blood Collection and Processing

1. 10 mL ethylene diamine tetraacetic acid (EDTA) or heparin vacutainer tubes.
2. Ficoll Hypaque (Histopaque 1077).

2.3. Surface Antigen Antibodies

Relevant monoclonal antibodies (mAbs) appropriate for FCM applications are used to identify cell populations being examined (the specificities of FCM-relevant mAbs are discussed especially in Chapters 9, 10, and 14). One must always verify that the antibody/antigen combinations being studied are robust to the procedure. For RT-PCR and PCR applications, use a biotinylated or other labeled antibody suitable for indirect detection. This is necessary, as in our hands, at least for FITC- or PE-labeled mAbs, either the fluorochrome or the conjugation of the fluorochrome to the antibody are destroyed by the heating required for PCR amplification. Even when employing an indirect approach, one must verify that the indirect label on the antibody is not destroyed by heating. We have never encountered problems when employing a biotin-labeled antibody. For direct hybridization (FISH), at temperatures under 55°C, use a direct-labeled antibody (*see* **Note 2**).

2.4. Cell Fixation and Permeabilization Reagents: Use One of the Following (*see* Note 3)

1. Ortho Permeafix.
2. Other commercial one-step fixation/permeabilization reagents.
3. Paraformaldehyde (1–4%) plus either 0.1% Triton X-100 or saponin (*see* **Note 4**).

2.5. PCR Reagents

1. GeneAmp *in situ* PCR core kit.
2. Digoxygenin dUTP or other bulky labeled nucleotide (*see* **Note 5**).
3. Forward and reverse primers (see recent reviews on primer design [***68–69***)].
4. PCR thermocycler.
5. Mineral oil (may not be necessary for some thermocyclers).

6. Sterile PCR reaction tubes.
7. Laminar flow hood.

2.6. RT-PCR Reagents

1. GeneAmp thermostable r*Tth* Reverse Transcriptase RNA PCR kit.
2. RNasin.
3. Digoxygenin dUTP or other bulky nucleotide (*see* **Note 5**).
4. Forward and reverse primers (primer design is discussed in several reviews **[*68–69*]**).
5. PCR thermocycler.
6. Mineral oil (may not be necessary for some thermocyclers).
7. Sterile PCR reaction tubes.
8. Laminar flow hood.

3. Methods

The methods described as follows are presented to serve as a framework for the design of FISH, or PCR(RT-PCR)/FISH protocols for cells in suspension from a variety of cell types and sources, ranging from cell lines to peripheral whole blood specimens. The steps are presented in order of operation, but each step should be optimized for specific applications. A single-cell suspension at a concentration of 1×10^6 cells/ml should be prepared before proceeding. Most procedures assume 1×10^6 cells per reaction are being used.

3.1. Antibody Staining

1. Add titer of mAb optimized for FCM and for this protocol directly to the cell pellet suspended in calcium and magnesium-free PBS with 30% bovine serum albumin (BSA) (*see* **Note 2**). The amount and type of blocking reagent utilized may vary with different specimens and antigen/antibody combinations. It is important to optimize antibody titer under the reaction conditions being used to ensure adequate cell staining. This can be done by preparing a mock hybridization reaction in which the cells of interest, labeled with several antibody concentrations, are carried through the entire procedure including fixation/permeabilization using all reagents and temperatures that will be used for the hybridization method, excluding only the probe(s). Analyze the samples by FCM to monitor for adequate signal to noise, observable by maximal separation of the positively stained cell population from the negative cells within the sample or other appropriate negative control. Performance of a quality control check performed by comparing the foregoing samples to similarly stained, but nonhybridized cell samples to verify cell recovery is also recommended, especially when studying heterogeneous populations. Detection of cytoplasmic antigens is also possible but staining needs to follow permeabilization or be incorporated into the fixation/permeabilization procedure.

2. Vortex the mixture gently followed by incubation at room temperature (protect from light) for 20 min (may need to be optimized for specific antibody and cell type), or manufacturer's recommended incubation time.

3.2. Fixation and Permeabilization

We have thoroughly tested Ortho Permeafix and found it to provide good results in general. However, in principle many of the commercial fixation and permeabilization reagents may be adequate but would need to be tested. Alternatively, a fresh 1–4% solution of paraformaldehyde prepared in DEPC-treated dH_2O may be used (*see* **Note 4**). When paraformaldehyde is used, a separate permeabilization step will need to be included following fixation. Typically a 0.1% Triton-X or Saponin treatment is used. Optimization of fixation and permeabilization parameters should permit adequate reagent access to intracellular target(s) while maintaining cellular integrity as to scattered light and antigen properties of interest. One is also advised to verify that there is no preferential cell loss at this stage, or any stage of the procedure, as described in the surface antigen antibody staining step. The following steps are recommended:

1. Wash cells in 1X PBS.
2. Vortex cells in residual fluid.
3. Proceed with fixation and permeabilization step(s) according to manufacturer's recommendations if using a commercial product. If using alternative reagents such as paraformaldehyde, determine the appropriate time, concentration, and temperature for your specific cell type. For some applications, optimization of commercial protocols for these parameters may also need to be done.
4. Vortex cells before incubation to prevent cell clumping.

3.3. PCR (optional—used when doing target amplification approach for DNA detection)

1. Wash antibody-stained, fixed, and permeabilized cell samples twice in 1X PBS.
2. Transfer 1×10^6 cells into each sterile PCR reaction tube with cap.
3. Pellet cells and remove any residual volume, being careful not to disturb the cell pellet.
4. Thaw all PCR reagents on ice, and prepare a master mix of solution, enough for $n + 1$ reactions, to allow for pipetting error. Substituting a bulky nucleotide such as digoxigenin dUTP as the substrate is important. Inclusion of a bulky nucleotide such as dig dUTP (or biotinylated dUTP depending on mAbs used) is necessary for *in situ* applications, as it is critical in aiding the retention of the amplified product inside the cell. As a general starting point for optimization, *in situ* PCR applications typically require longer annealing and elongation times, increased enzyme concentrations (typically 2X), and increased magnesium concentrations as compared to standard PCR reactions with purified nucleic acids

with other parameters usually being similar. Individual PCR profiles will vary according to primers and targets used, and will have to be optimized individually for each procedure. In general, PCR programs consist of a short denaturation stage, followed by a primer annealing step, and a primer extension step. The number of cycles required will need to be empirically determined as to when adequate target product has been generated.

5. After amplification, the samples can be transferred into 12 × 75 mm or 15 mL Falcon tubes before proceeding with the prehybridization step.
6. Optimize the foregoing parameters (recommended) through alternate methods such as gel electrophoresis. Run the isolated nucleic acid, extracted from the cells that have been amplified, on a gel to verify the appropriately sized product(s) have been generated. This is a good quality control to perform at least once early on in the workup of a new application. In addition, other molecular techniques such as Southern hybridization or sequencing can also be performed to further document the specificity of the amplification reaction (*see* **Notes 6** and **7**).

3.4. RT-PCR (Optional—used for target amplification approach for RNA detection.)

1. Wash antibody-stained, fixed, and permeabilized cell samples twice in 1X PBS.
2. Transfer 1×10^6 cells into each sterile PCR reaction tube with cap.
3. Pellet cells and remove any residual volume, being careful not to disturb the cell pellet.
4. Thaw all RT-PCR reagents on ice, and prepare a master mix of solution, enough for $n + 1$ reactions, to allow for pipetting error. Substitute a bulky nucleotide such as digoxigenin dUTP as the substrate is important. Including a bulky nucleotide such as dig dUTP during both the reverse transcription and amplification step is crucial for *in situ* RT-PCR applications, as it was for *in situ* PCR. RT-PCR applications in general also require longer reverse transcription times, as well as longer annealing and elongation times. As with *in situ* PCR, increased polymerase concentrations (typically 2X), and increased magnesium concentration are again typically required. Individual RT-PCR profiles will vary according to primers and targets used, and will have to be optimized individually for each procedure, as will the number of PCR cycles used. In general, RT-PCR programs consist of a reverse transcription step, short denaturation stage, followed by repeated primer annealing and primer extension steps. After the reverse transcription stage, additional reagents need to be added to begin the PCR amplification process, refer to the product insert.
5. After amplification, the samples can be transferred into 12 × 75 mm or 15 mL Falcon tubes before proceeding with the prehybridization step.
6. As was described for *in situ* PCR, optimization of all the above parameters can be confirmed through alternate methods such as gel electrophoresis. Nucleic acids isolated from cells that have been amplified can be run on a gel to verify that the appropriately sized product(s) have been generated. As stated, this is a good qual-

ity control to perform at least once early on in the workup of a new application in addition to other molecular techniques such as Southern hybridization or sequencing to further document the specificity of the amplified product (*see* **Note 6** and **7**).

3.5. Prehybridization Step

1. Wash cells 1X in PBS, centrifuge, aspirate, and vortex.
2. Wash cells in 2X SSC, centrifuge, aspirate, and vortex gently to suspend cells in residual fluid.

3.6. Fluorescent *in situ* Hybridization (FISH)

The stringency, or specificity, of base-pair matching in an annealed probe/target complex is controlled by varying the temperature of the hybridization reaction relative to the melting temperature of probe/target complex under the experimental conditions. Thus, exact hybridization reagent compositions vary depending on probe/target sequence, desired working hybridization temperature, and degree of stringency (specificity) required. Including a carefully chosen blocking reagent(s) in the hybridization buffer is critically important (*see* **Note 8**). However, inclusion of blocking reagents which are standard for Southern or Northern blot analyses, such as Denhardt's, or use of dextran sulfate to reduce hybridization time are not needed, and generally increase the background signal, making positive signal detection problematic. Additionally, the viscosity of these solutions makes centrifugation of cells difficult. Some general guidelines for designing hybridization reagents and adjustment of hybridization conditions are presented as follows.

3.6.1. Hybridization Buffer

A typical hybridization buffer might include 2–5X SSC, 20–50% molecular-grade formamide, and a blocking reagent such as 0.5 mg/mL sheared salmon sperm DNA, brought to volume in DEPC-treated dH_2O. The SSC and formamide concentrations will depend on the probe/target sequence ("inherent melting temperature") and the desired temperature of hybridization at which one wants to work (decreasing salt concentration and increasing formamide concentration decreases the melting temperature of nucleic acid complexes). Performing hybridization at lower temperatures is not only technically easier in the laboratory but is also more compatible with the thermal stability of many of the other reagents used, such as labeling fluorophores for both mAbs and nucleic acid probes. Working volumes will need to be determined, as will the hybridization temperature and the length of time for the hybridization. Volumes depend mainly on the final concentration of the probe(s) desired, and although important, are less critical than optimization of the actual probe to target (cell number × number of copies per cell) ratio for your system. Optimal

probe to target ratios result in adequate, or preferably maximized, signal to noise for a given set of optimal temperature and time of hybridization. Obviously, positively hybridized samples need to have intense enough specific signal above background and nonspecific fluorescence to resolve them from negative cell populations (*see* **Note 8**).

1. Add hybridization buffer to cells in suspension (typical volume 25–50 μL).
2. Add probe, or probe cocktail to the cell suspension.
3. Incubate at appropriate temperature for your probe/target sequence melting temperature under the defined buffer conditions. Generally, hybridization temperature is at or a few degrees below the melting temperature (*see* **Note 9**).

3.6.2. Probe Design

If performing target amplification (PCR or RT-PCR), a single fluorescently labeled probe specific for the amplicon of interest is usually adequate. Labeling the probe for indirect detection, such as biotin labels, can also be employed. Probes need to be of very high quality and should be made with as much care as described herein for making individual probes that are to be utilized in a cocktail in direct FISH approaches. Making and verifying quality of one probe, however, is far easier than doing the same for the multiple probe approaches described as follows.

In contrast, to obtain adequate sensitivity (detection of less than several hundred copies of a sequence of interest) in direct FISH approaches, one will need to employ some type of signal amplification technique. Although signal amplification from one, or a limited number, of probes can be done, few examples exist for this approach in FCM detection of low copy number targets. An alternate approach is to employ a large number of directly labeled oligonucleotide probes in a "cocktail" that in aggregate hybridize to, or span, the majority of a sequence of interest within the cell. One could employ labeling of a construct containing an insert homologous to a gene of interest, as previously mentioned. The latter technique has been employed for high copy number target sequences. However, in many instances this may not be optimal for detection of low copy number targets, as it will produce a heterogeneous mixture of fairly large probes (100–400 base pairs) with variable melting temperatures. We have obtained maximum sensitivity employing short oligonucleotide probes in cocktails of 50–300 probes. Employing synthesized oligonucleotide probes allows one to tightly control the characteristics of the individual probes in terms of melting temperature and other characteristics described shortly. The design, manufacture, and screening of these large cocktails of oligonucleotide probes can be costly and time consuming, as each probe must meet stringent quality requirements described shortly. However, for higher copy number targets (>500–1000 copies)

a smaller number of probes may be utilized, making this approach more practical. For example, as few as five oligonucleotide probes have been employed in detection of high-level viral RNA targets ***(23,26)***. At the present time, our experience would favor the following recommendations, or considerations, in constructing these cocktails:

3.6.2.1. Directly Labeled Probes

Phosphoramidite labeling chemistry works well. Other labeling chemistries may work just as well, but testing and possibly further development may be needed. Specific fluorophore used is also important. Consult your probe manufacturer for more specific recommendations, however, keep in mind that few synthesis companies have experience in the use of their probes for *in situ* analyses with cells in suspension. Thus, at the present time, you must carefully screen the performance of your probes for use in flow cytometric FISH assays (*see* **Note 8**).

3.6.2.2. HPLC Purified

Critical to ensure all probes are "clean," have similar nonspecific binding, and that all are labeled identically. Even minor nonspecific binding for each probe can soon sum to levels that, in aggregate, can swamp specific signals. Obviously, any inconsistency in labeling will be severely detrimental to the final signal to noise of your measurement.

3.6.2.3. Similar T_M

The narrower the range the better, typically less than a 5°C spread in melting temperature is best, although a tighter (2 or 3°C) separation is optimal. This is an important point, as control of stringency will be impossible if all of your specifically bound probes are not melting on and off their respective target sequences at the same temperature. As mentioned earlier, use of probes whose T_M is less than 55°C under the specific salt and formamide concentrations used will provide more flexibility with fluorophores used on the directly labeled mAbs and probes. The probes used should have no self-complementarity or homology to endogenous cellular sequences in the cell populations to be studied other than to the sequences of interest. All probes must have low and similar nonspecific binding characteristics, which are obviously aided by ensuring high purity and consistent labeling. In determining the temperature of hybridization, one must take into account the level of stringency (specificity or number of mismatched bases which are acceptable) required for the analyses to be performed. In general, hybridizations are done a few degrees below the melting temperature of the probe target complex under the experimental conditions being studied, as probe/target complex having

mismatches will have a lower melting temperature (T_M) than ones that are perfectly basepair matched. In determining the T_M, one must take into account the GC/AT ratio of the probe target complex as well as the formamide and salt concentrations. Inherent T_M can be calculated using any of the readily available commercial software programs such as Oligo, or shareware programs available on the internet, such as Primer. Using the nearest-neighbor method calculation model is recommended, as it takes into account GC/AT content as well as specific nucleotide position or order in the sequence. A rough calculation for the dependence of the melting temperature of a probe/target complex on formamide concentration is as follows:

$$T_M - (\%\text{formamide})\ (0.63) = T_H$$

where T_M is the melting point of each individual probe and T_H is the hybridization temperature to be used at a specific formamide concentration. The change in melting temperature with increasing formamide concentration is actually dependent on GC/AT ratio with 0.63 being an average of the change for a GC basepair versus an AT basepair; however, in most instances this estimate is more than adequate for determining a starting point for the hybridization reactions. For the probe cocktail, one uses the average T_H of all the probes. As one lowers the salt concentration, one lowers the melting temperature of nucleic acid complexes, and thus, one must also take into account the salt concentration to be used. Most programs that automatically calculate melting temperature calculate this at a user-input salt concentration. Once you have determined the average T_H of your probe cocktail in the buffers used, hybridizations are carried out at a temperature of 5–8°C below the T_H. This temperature is a starting point, and the best hybridization temperature should be selected based on empirically optimizing the signal to noise of the detected FISH signal. For a more in-depth discussion of these principles for isolated nucleic acids, of which many of the same principles apply *in situ*, the reader is referred to the literature ***(70)***.

3.7. Posthybridization Wash

1. A wash is performed immediately after hybridization at conditions below the melting temperature of the probe–target complex, mainly to remove any unbound excess probe in solution.
2. 2–5X SSC (same as hybridization buffer), 0.1X Triton-X or Saponin.
3. Wash with excess volume.

3.8. Stringency Wash

A stringency wash is generally performed under conditions such that one is several degrees closer to the T_M of the probe/target complex than during the

hybridization, thus increasing the specificity of the bound probe. Frequently, this is accomplished without formamide in the buffer by slightly raising the temperature and dramatically lowering the salt concentration (tenfold lower in the following example). This step may be performed one or two times.

1. 0.2–0.5X SSC, 0.1 X Triton-X or Saponin.
2. Wash in excess volume, to be performed at the hybridization temperature, or slightly higher.
3. A brief incubation (10–15 min) is recommended.

3.9. Secondary Reagent Addition (For biotinylated, or other indirect MoAbs and/or probes.)

1. Add optimized amount of secondary reagent (may require an independent titering for optimization of signal).
2. Incubate length of time and at temperature which gives optimum signal (generally for fluorescently labeled avidin detection of biotinylated mAbs, 20 min at room temperature works well) or as recommended by the manufacturer.

3.10. Final Suspension

1. 1X PBS calcium chloride, magnesium chloride, and sodium azide free.
2. Add an optimized concentration of trypan blue, or similar dye, just before analysis. Typical final concentrations are 1–2 µg/mL.

3.11. Cell Analysis by FCM

Standardize the instrument as per your laboratory's individual quality control procedures (*see* also Part III of this volume). Care should be taken that appropriate setup of color compensation is done under the instrument settings and fluorophores used for analysis. Prepare an analysis protocol to record all relevant parameters. Minimum suggested is a forward vs. side scatter (to verify cellular integrity), fluorescence vs. side scatter, and appropriate single or dual parameter fluorescence histograms. Both gated and ungated data should be collected so that all background or nonspecific staining is examined. Please note that fixation, heating, and some reagents used in this procedure will affect scatter characteristics and background fluorescence levels. Routine use of known negative as well as known positive controls to set up your experimental protocols are mandatory, as settings will vary greatly from other procedures. Ideally, each sample will inherently have both negative and positive cell populations in it for comparison. In some cases, background autofluorescence will be significantly increased and additional methods to reduce this interference, such as inclusion of trypan blue in the analysis buffer ***(71)***, may need to be done. Trypan blue can, to some extent, also reduce nonspecifically bound fluorescence signal ***(71)***. An example of simultaneous cell surface antigen staining

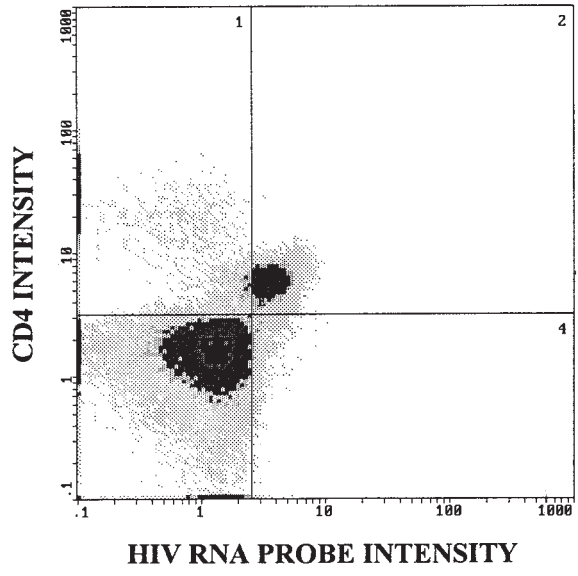

Fig. 3. Bivariate histogram of anti-CD4 staining vs. HIV RNA probe intensity for peripheral blood mononuclear cells isolated from a HIV positive patient. Note that the positive hybridization signal in the dim $CD4^+$ cells (monocytes) as compared with either the bright $CD4^+$ cells (helper cells) or the $CD4^-$ cells in the sample. Similar studies using CD14 antibodies verified the HIV RNA positive result in the monocyte population (*see* **ref. *22***).

(anti-CD4 PE labeled) and HIV RNA detection employing a cocktail of FITC-labeled oligonucleotide probes is shown in **Fig. 3**.

4. Notes

1. Design of probe cocktails, as in the case of signal amplification, is essential. Use a reliable, high-quality manufacturer, and work closely with them to ensure that all quality control standards have been met.
2. If directly labeled cell surface mAbs are to be used during direct hybridization, they should be tested for stability under the temperatures and in the presence of reagents used in the procedure. This will include the fluorophore itself, the fluor to antibody linkage, and the antibody–antigen binding and complex stability after fixation.
3. Fixation and permeabilization are critical for allowing reagents to pass through the cell membrane while maintaining cellular antigen targets of interest and cellular integrity. Conditions will vary by cell type.
4. If paraformaldehyde is being used as opposed to commercial preparations, it must be made fresh and pH adjusted the day of use.

5. If PCR and/or RT-PCR are being employed, use a bulky nucleotide such as digoxygenin dUTP to retain the amplified product within the cell. Verify that all product is being produced and retained *in situ* by running aliquots of both the supernatant and lysed cells after amplification on a gel.
6. Additional negative controls for PCR and RT-PCR include use of irrelevant primers, or no primers, and irrelevant probe (stringency matched) for the FISH detection of the amplified product. One may also leave out the RT enzyme in the case of RT-PCR. Elimination of *Taq* can also be done but will not reflect the background arising from the repair activity of *Taq*. Negative control cocktails are theoretically possible but must be perfectly stringency matched and have identical nonspecific binding characteristics to the positive or test cocktail. They are equally as expensive and difficult to manufacture, as the test cocktail and are frequently impractical. In these cases, one must rely on negative and positive control cell lines and on comparison to inherently negative or positive cells within heterogeneous patient samples.
7. To verify amplification in PCR and RT-PCR, one may use an irrelevant primer/probe pair that is present in all cells, such as HLA-DR. This ensures that amplification took place in all cells within the sample.
8. Nonspecific binding and background measurements should be made with appropriate cellular controls processed in the same way as the experimental sample. This should include not only negative cells, but ideally one will have a negative cell population within a heterogeneous sample for comparison. Additionally, a positive control should be run.
9. Stringency requirements will change with alterations in any of the hybridization reagents including the probe. Changes should be compared to previous reagents in a known, reproducible, controlled system.

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12

Quantitative Fluorescence Cytometry

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1. Introduction

The phenotypes useful in distinguishing normal and neoplastic leukocytes are often identified by fluorescence staining reactions detected on flow cytometers. These reactions were originally observed by fluorescence microscopy, and cells were classified by human observers as simply negative or positive, with the positive cells sometimes distinguished as dim or bright. These terms are still used in analyzing flow cytometry (FCM) results. However, recent advances in our understanding of fluorescence signals from stained cells ***(1)*** now permit the translation of terms like "dim" and "bright" into real mass units of fluorescence intensity, a process that we call quantitative fluorescence cytometry (QFCM). Although the translation is not yet exact and certain technical details remain to be resolved, a general understanding of QFCM is now accessible and helpful in interpreting staining patterns.

The importance of fluorescence intensity has been emphasized in a number of recent reviews on the use of flow cytometry in evaluating hematologic malignancies ***(2–6)***. One reason for this recent emphasis is our increasing awareness that neoplasia can alter the magnitude of cell protein expression even if it does not cause the complete absence of a normal protein or the presence of an unexpected one. Because the fluorescence intensity of stained cells reflects (indirectly) the extent of expression, a quantitative approach to fluo-

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rescence can provide much more extensive information about cell properties than a simple dichotomy of negative and positive.

QFCM presumes the ability to assign numeric values to fluorescent signals from stained cells. Indeed, a number of reports now include specific numeric values in units of fluorescence that we describe in **Subheading 1.1.** However, a more general aspect of QFCM will be familiar to anyone who looks at the fluorescence histograms from flow cytometric phenotyping. These histograms are most often depicted as a series of two-dimensional ("bivariate") dot plots or contours that, taken as a whole, provide a "phenogram" ***(7)*** or "phenotype map" ***(8)*** of the cell mixture analyzed. Just as a scale of miles makes a roadmap more useful, the scale of fluorescence intensity provided by QFCM can greatly enhance the ability to compare patterns and recognize aberrant phenotypes that may reflect malignant transformation. Although our discussion will be restricted to FCM because of its widespread use, the basic tenets of QFCM are applicable to other forms of fluorescence cytometry that are just now emerging into clinical use, such as laser scanning cytometry ***(9)*** and volumetric capillary cytometry ***(10)***. We hope the reader will see that a simple understanding of QFCM can complement and extend the visual impressions that are so useful in evaluating the phenotype patterns FCM provides.

1.1. Quantitative Units of Fluorescence Intensity

The amount of fluorochrome on stained cells is quite low, generally in the range of a few thousand to a few million molecules. We thus find it convenient to express units of fluorescence intensity (FI) in terms of molecules rather than gross units like grams or moles. Properly calibrated microparticle standards for flow cytometry accomplish this by expressing their FI values in units called Molecules of Equivalent Soluble Fluorochrome, abbreviated as "MESF." We will not delve into the technicalities of MESF here (*see* ***11*** for details) except to note that MESF values from different fluorochromes are not comparable so that each fluorochrome must be calibrated in its own MESF units. The relationship between fluorescence intensity expressed as MESF units and the amount of the fluorescent conjugate staining the cells is discussed as follows.

1.2. FI and the Window of Analysis

The strength of fluorescence intensity measured on each stained cell or particle is depicted on fluorescence histograms. Each histogram is divided into a fixed number of discrete channels (usually 64, 256, or 1024). Events in the lowest channel (designated as the "zero channel") have the weakest signals and events in the highest channel (the "maximum channel") the strongest. Because events must lie between the lowest and highest channels, the histogram is essentially a "window of analysis" ***(11,12)*** for FI.

Most clinical flow cytometers use electronics that can amplify fluorescence signals as much as 10,000-fold, thus the highest channel in a fluorescence histogram will represent MESF values about 10,000 times greater than MESF value of the lowest channel. This degree of amplification is necessary to detect the weakest staining reactions. Cytometrists often refer to "four decades of dynamic range," as 10 raised to the fourth power gives the factor of 10,000. Because of this wide dynamic range, the fluorescence histogram is best depicted on a logarithmic ("log") scale so that the dimmest and brightest events can be appreciated on the same display. In the usual log histogram display, the first quarter of the histogram represents the first decade, the second quarter represents the second decade, and so forth. The dimmest events, typically reflecting the background fluorescence of unstained cells, occupy the first decade, and events in the higher decades represent increasing levels of staining intensity.

1.3. The Common Window of Analysis

The four-decade logarithmic histogram where the autofluorescence of unstained lymphocytes is visible in the first decade has been called a "common," "typical," or "unified" window of analysis (*see* **Fig. 1**). In practice, some applications will require the window to be shifted to a dimmer range, or, especially for immunophenotyping leukemias and lymphomas, to a brighter range. The adjustment of voltage to the photomultiplier tube (PMT) controls placement of the window: higher voltages move the window to the "left" (i.e., dimmer regions) and allow negative cells to be resolved from the zero channel, whereas lower voltages move the window to the "right" (i.e., brighter regions) and allow brightly stained cells to be resolved from the highest channel. (This concept is illustrated in the Internet training program at www.fcstd.com—select "animations" on the left side of the home page, then "PMT" to see the window move as a function of PMT voltage.)

1.4. MESF Calibration

1.4.1. General Aspects

Fluorescence histograms are calibrated in MESF values by analyzing the proper microbead standards and determining the relationship between histogram channel numbers and MESF values ***(13)***. Because a wide variety of microbead standards are available, and different types are suitable for different purposes ***(14)***, the laboratory should be certain that valid MESF standards are used for quantitative calibration. Although there is not yet a single standardized approach for calibrating fluorescence on flow cytometers, the protocol ***(2)*** that emerged from the U.S.–Canadian consensus recommendations on the

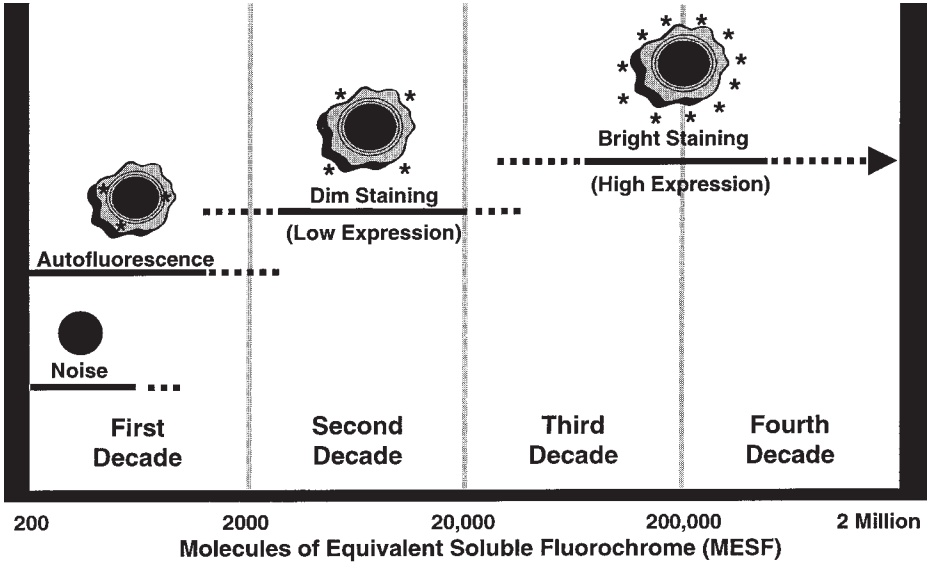

Fig. 1. Schematic of an idealized window of analysis for phenotyping circulating leukocyte surface receptors with FITC-labeled monoclonal antibody conjugates. Fluorescence intensity is shown on a logarithmic scale encompassing a range of four decades (equivalent to 10,000-fold difference from the lowest to the highest channel). The equivalent MESF values are shown for each decade. To achieve this window, the photomultiplier voltage should be set just high enough to bring the distribution of noise (determined by analyzing certified blank microbeads) almost fully on scale (i.e., so very few events accumulate in the lowest channel). At this setting, the highest channel in the window represents about 2 million MESF, a value large enough to keep the brightest staining on scale for the most common surface receptors on normal circulating leukocytes.

immunophenotypic analysis of hematologic neoplasia by flow cytometry ***(4)*** is probably the most detailed and useful. Further refinement through consensus is soon forthcoming: the National Committee for Clinical Laboratory Standards (NCCLS) recently convened a subcommittee to promulgate a guideline for quantitative fluorescence calibration that would be applicable to instrument manufacturers, reagent suppliers, and laboratories.

1.4.2. The MESF Calibration Curve

In practice, the relationship between FI and histogram channel is essentially linear in the midrange of the histogram, so the calibration curve can be depicted as a straight line represented by a simple equation (*see* **Fig. 2**). MESF values from this midrange line can be extrapolated down to the zero channel and up to

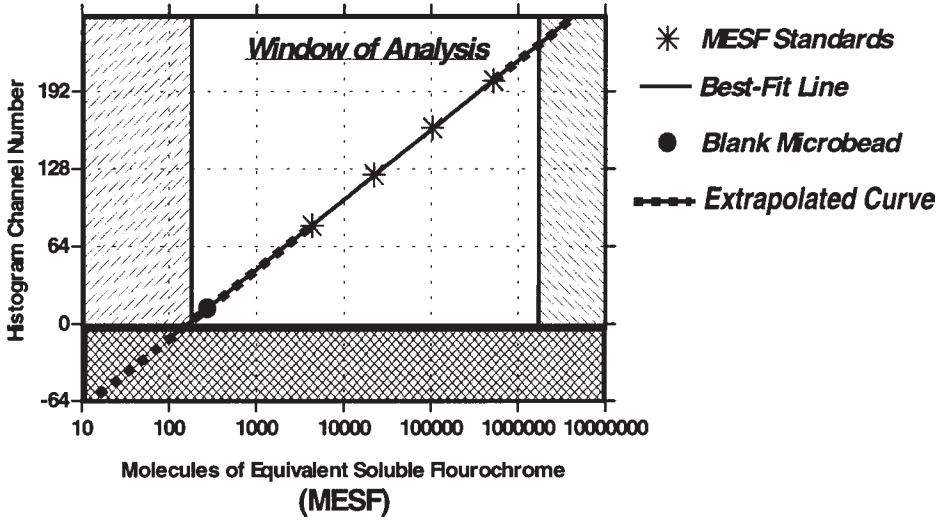

Fig. 2. A typical calibration curve relating the fluorescence intensity in MESF units (*X*-axis) to the fluorescence histogram channel number (*Y*-axis). The curve is constructed by a simple linear regression of the four data points from the MESF standards to obtain the best-fit line. The calibration curve is extrapolated below and above the highest standards to obtain hypothetical MESF values corresponding to the zero channel and to the maximum channel, respectively. In this example, the zero channel value is about 200 MESF and the maximum channel value is about 2 million MESF. The window of analysis is the region between the zero and maximum channels.

the maximum channel. These extrapolated values are approximations (as linearity fails at the extremes of the measurements), but they provide a useful benchmark that should be quite consistent between analyses if the cytometer is functioning and calibrated properly.

The window of analysis expressed in quantitative terms is the range of MESF values from the zero channel to the maximum channel of the fluorescence histogram. **Figure 1** schematically depicts a typical window of analysis from a flow cytometer optimized for phenotyping peripheral blood lymphocytes that have been stained with monoclonal antibodies (mAbs) conjugated to fluorescein isothiocyanate (FITC). The photomultiplier voltage has been adjusted just high enough that blank microbeads do not accumulate in the zero channel, so they appear on scale in the lowest ranges of the histogram. Because the FI of blank microbeads approximates the background noise, this setting resolves the noise from the zero channel and ensures that sensitivity will not be lost due to real (although weak) fluorescence signals accumulating in the zero channel. At this setting, the highest channel contains events with FI values in the range of a

few million MESF, higher than the FI from normal peripheral blood lymphocytes stained for most cytoplasmic or cell surface receptors. Other cell lineages, activated cells, and neoplastic cells may stain more brightly and require adjustment of photomultiplier voltage so that noise (or even weak fluorescence) is restricted to the zero channel to allow the brightest events to appear on scale.

1.4.3. Lymphocyte Phenotypes and MESF Values

Actual data for selected phenotypes and MESF microbead standards (*see* **Fig. 3**) demonstrates the range of staining commensurate with background, dim, moderate, and bright FI in the typical window of analysis for FITC-labeled antibodies. Background fluorescence from cells exceeds the noise from blank microbeads because cells contain natural fluorochromes. This autofluorescence is low in the resting population of peripheral blood lymphocytes, averaging around 1000 MESF for most analyses with clinical cytometers. We can see from the distribution of autofluorescence that any lymphocyte stained at a level of at least 2000 MESF will be distinctly separated from the background. This value establishes a practical lower limit of detection with high (>99%) specificity for true staining; individual resolution of stained and unstained cells will not be possible below this limit. Autofluorescence can be very bright (above 10,000 MESF) in highly activated lymphocyte populations and in certain granulocytes, especially eosinophils. This degree of autofluorescence may impair the detection of low-level staining.

Staining for the cell surface receptor CD4 depicted in **Fig. 3** demonstrates both dim staining (in monocytes) and moderate staining (in the helper subpopulation of T cells). The monocyte population stained with CD4-FITC represents a well-known dim distribution first defined by comparison with the brighter CD4 staining on helper T cells ***(15)***. Some overlap with autofluorescence is apparent; we cannot be certain whether these dimmest monocytes are weakly stained, highly autofluorescent, or both. However, a clear distribution of positive staining can be seen from about 2000 MESF to about 15,000 MESF, showing a broad range of staining within the population. The helper T cells stained with CD4-FITC show a characteristic distribution: discrete, moderately bright, and highly symmetric on a log scale. The median value of the distribution is 44,000 MESF and the entire population encompasses around 30,000–70,000 MESF. The CD45-stained lymphocytes are brighter: their median value is about 225,000 MESF and they span from about 140,000–500,000 MESF. The CD38-stained plasma cells are the brightest population. Only part of the population is visible in the window, as most of the cells have accumulated in the maximum channel; this is a good example of the need to shift the window of analysis to the right by reducing the PMT voltage.

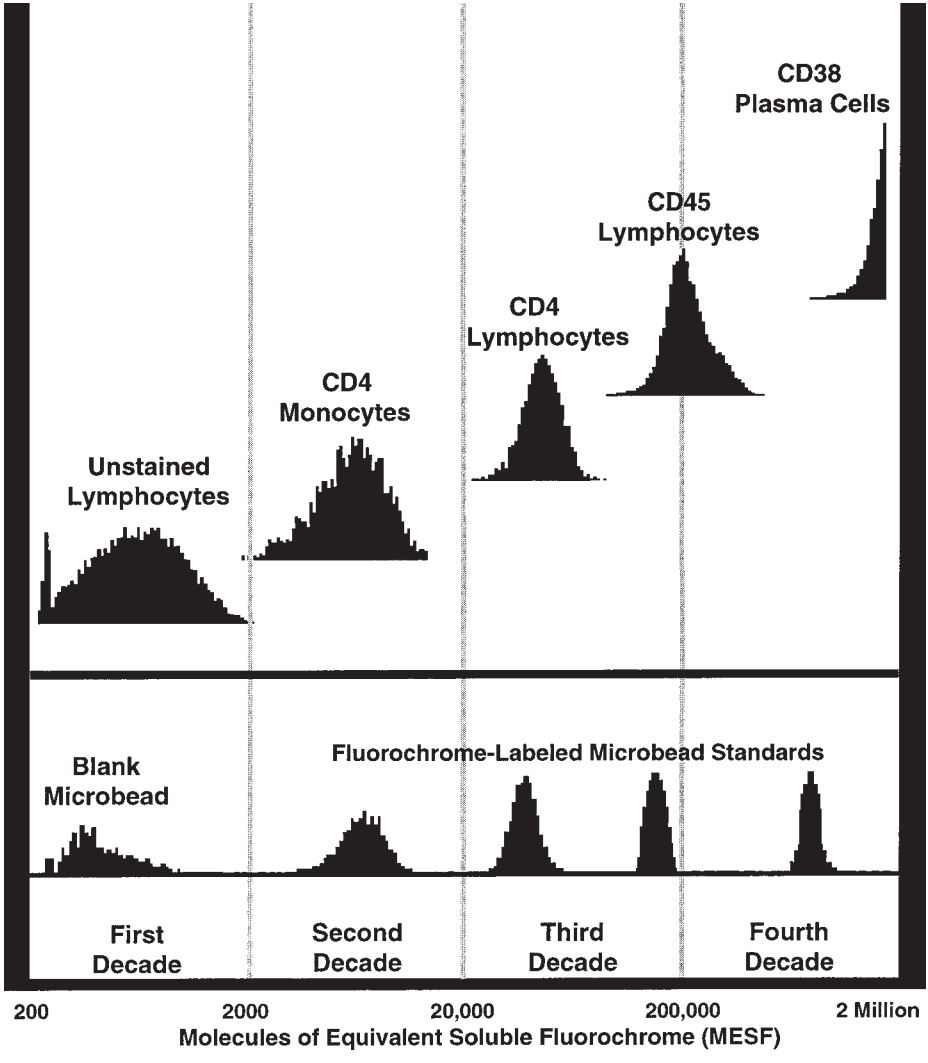

Fig. 3. The same window of analysis shown in **Fig. 1** with actual data from normal peripheral blood leukocytes stained with FITC conjugates (upper region) and from MESF microbead standards labeled with FITC (lower region). The microbead standards correspond to the data points shown on **Fig. 2**. The fluorescence photomultiplier has been adjusted just high enough to keep certified blank microbeads from accumulating in the zero channel. At this setting, autofluorescence causes unstained lymphocytes to occupy most of the first decade. The brightest staining is shown by CD38 on plasma cells, many of which accumulate in the maximum channel because their fluorescence is brighter than 2 million MESF.

1.4.4. Using Fluorescence Intensity to Quantify Ligand Binding

Knowing the assigned MESF value (interpolated or extrapolated from the standard curve) for any point in the window of analysis allows us to explore receptor expression on cells and the chemistry of the receptor–ligand interactions responsible for fluorescent staining. By convention, the term "receptor" will always refer to the particulate (cell or microbead) molecule and the term "ligand" will refer to the fluorochrome–protein conjugate molecule (usually a monoclonal antibody) used for staining.

1.4.4.1. Antibody Binding Capacity (ABC)

Using **Fig. 3** as a model, we will first consider the labeled ligands. These FITC-conjugated mAb molecules actually supply the fluorescence signal, so the MESF value is really quantifying their binding to the stained cell. Because most conjugates like these are antibodies directed against cellular antigens, the term "antibody binding capacity" (ABC) is often used to denote the number of conjugate molecules bound to a stained cell when virtually all receptors are bound ***(11)***. The ABC is thus an indirect indicator of receptor expression. The ABC value is related to the MESF value through a conversion factor called the "effective *F/P* ratio" of the conjugate; it is analogous to the "specific activity" of a molecule labeled with a radioactive isotope. In practice, effective *F/P* must be determined by measuring the MESF value of a known concentration of conjugate. This can be accomplished by solution fluorimetry or by FCM, the latter using microparticles that bind a known quantity of conjugate. The conversion from MESF to ABC is accomplished by multiplying the MESF value times the effective *F/P* ratio:

$$\text{ABC} = (\text{MESF}) \times (\text{effective } F/P \text{ ratio})$$

The effective *F/P* ratio is influenced by the amount of fluorochrome used to label the conjugate and a number of other factors that diminish fluorescence, collectively referred to as "quenching." Although the effective *F/P* ratio varies from conjugate to conjugate, it is possible to estimate a general value for most of the FITC conjugates used for FCM because they have a rather narrow range of fluorescein conjugation and quenching that results in an effective *F/P* ratio close to 1. To simplify this discussion, we will assume an effective *F/P* ratio of exactly 1.0 for the conjugates used in **Fig. 3**. Thus, the MESF value of a cell labeled by an FITC antibody conjugate may be presumed equal to number of antibody conjugate molecules staining the cell. The CD4 lymphocyte peak shown in **Fig. 3**, encompassing 30,000–70,000 MESF, would therefore represent cells binding 30,000–70,000 CD4–FITC conjugate molecules. Direct binding measurements of this population showed an average ABC value of about

48,000 molecules ***(16)***, consistent with our assumptions. As another example of the 1.0 approximation for effective *F/P* ratio, note that the CD45 population has an average MESF of about 200,000 MESF, almost identical with published ABC values ***(12,17)***.

We emphasize that, although the approximate value of unity for the effective *F/P* ratio applies to most FITC antibody conjugates used for direct staining in FCM, it is unlikely to be appropriate for most other fluorochrome conjugates. Ideally, reagent suppliers would measure effective *F/P* ratios directly on each lot of reagent and provide these values to the laboratory.

1.4.4.2. ABC as a Measure of Cell Receptor Expression

Ultimately, we would like to know how ABC values compare with the actual number of receptors on the cell. This relationship is complicated by several factors. The first consideration is whether enough fluorescence conjugate is used to achieve saturated binding, so that virtually all the available receptors are labeled. For the conjugates used in **Fig. 3**, prior titration experiments had demonstrated that the concentrations of conjugate were indeed saturating. (We note that absolute saturation is not possible, as the chemical equilibrium of the binding reaction always includes some dissociation, but the equilibrium constants of conjugates used for diagnostic purposes should be high enough to provide virtual saturation with adequate concentrations of reagents.)

The next consideration is the valency of binding between the conjugate and the cell receptor. Because each antibody molecule has two binding sites, each conjugate molecule could conceivably bind two cell receptors. In that case, the number of receptors would be twice the ABC value. This "bivalent" relationship has been elegantly demonstrated for lymphocyte binding of a CD4 conjugate labeled with phycoerythrin (PE), where the ABC of conjugate from intact antibody averaged about 48,000 molecules, whereas the ABC of conjugate from the single-armed Fab averaged about 95,000 molecules ***(18)***. Although we do not have direct evidence for bivalent binding with the CD4-FITC conjugate used in **Fig. 3**, it is strongly suggested by the agreement between the ABC value we obtained (48,000) and the range of ABC values obtained with the CD4–PE conjugate (46,000–50,000). We can therefore be confident that the average peripheral blood CD4 T cell expresses about 95,000 CD4 molecules. However, the valency factor is uncertain for most other conjugate–receptor interactions.

A third consideration is whether the receptor epitope recognized by the antibody is readily available for binding. Other cell surface molecules can caused so-called "epitope masking," in which case the ABC value will be lower than the number of receptors, or even undetectable. An example of false

negatives due to epitope masking occurred early in the use of FCM to count CD4 lymphocytes. One of the CD4 mAb conjugates in common use at that time failed to stain CD4 lymphocytes in a small subset of individuals even though conjugates from other CD4 monoclonal antibodies stained the lymphocytes as expected. The CD4 epitope recognized by this particular mAb (OKT4) was in the region where the receptor penetrated the cell membrane, and an autosomal recessive trait unrelated to the primary structure of the CD4 protein caused blockage of antibody binding ***(19)***. This CD4 epitope masking may even be associated with the pathogenesis of acute myelogenous leukemia ***(20)***. For the leukocyte populations and stained with the reagents shown in **Fig. 3**, epitope masking does not appear to be a significant problem in most conjugates selected for clinical applications.

The final consideration is whether nonspecific binding (NSB) could account for staining, which would give a mistaken impression of receptor expression. This is not much of a problem on normal resting peripheral blood lymphocytes, but leukemic cell populations are not so predictable, and some may show a large degree of NSB. So-called "isotype controls" or "subclass controls," conjugates of the same fluorochrome and antibody isotype (e.g. IgG1a, IgG1b, IgG2a, IgM), but having no specificity for cell receptors, are recommended to detect or rule out NSB ***(21)***. Such isotype controls are useful but not ideal, as even with the same isotype, different mAb conjugates may differ in their extent of NSB to cells, depending on both the antibody and the fluorochrome. The best control, although rarely used, is a duplicate assay in the presence of a large excess of soluble receptor. Because the excess soluble receptor will compete for specific binding with the cell surface receptor, any residual cellular fluorescence must be due to nonspecific binding or autofluorescence. Another option is competition with unlabeled soluble antibody, which is more readily available than soluble receptor. However, this control cannot rule out specific binding by other cell receptors (such as Fc receptors) to alternate sites on the antibody conjugate molecule.

The several factors that complicate the relationship between ABC and receptor expression may seem daunting, but a few considerations applicable to direct staining with fluorescent conjugates simplify the relationship. First, an adequate concentration of conjugate will ensure near saturation of receptor–ligand binding sites; conjugates designated for diagnostic use should be supplied with documentation of the concentrations required for saturation, and the end user should confirm this with titration studies. Second, the mAbs used in conjugates should have been selected by the supplier to avoid the possibility of false negatives due to epitope masking, although again the cytometrist must remain alert for any capricious failure to stain, perhaps induced by alterations in malignant cells. Third, differences in the valency of antibody binding pro-

duces at most a twofold range of uncertainty, a relatively small imprecision when considering the thousand-fold range of common receptor expression. Finally, non-specific binding rarely interferes with moderate to strong staining reactions (though it can certainly complicate weaker ones). On the whole, the ABC value may be taken directly as a reasonable estimate of receptor expression for typical staining reactions.

1.5. Alternative methods of FI calibration for Quantifying Fluorescent Conjugate Binding

Other approaches to FI calibration on cytometers are available that do not require MESF or effective *F/P* values to measure binding capacities. In these systems, standards calibrated directly in ABC values (rather than MESF values) are used to construct a calibration curve directly relating the ABC value to the FI value. For direct staining, the same conjugate-binding microbead standards used to determine the effective *F/P* ratio can be used to construct an ABC calibration curve ***(13)***. The actual ABC values of the microbeads may vary between conjugates and so the assigned ABC values must be confirmed. Other investigators have used CD4 expression on T cells as a biological standard for measuring the ABC of PE conjugates. By assuming a nominal value of 50,000 ABC for CD4 T cells, a constant relating histogram channel number to ABC is derived and applied to other PE conjugates with the same 1 : 1 molar *F/P* (see the Web site http://cyto.mednet.ucla.edu, select "Protocols" then "CD38 Fluorescence Intensity Quantitation"). For measuring ABC by indirect staining (i.e., binding an unlabeled antibody to cell receptors then staining with a second antiantibody conjugate), another system of calibration has been developed and applied extensively to hematopoietic cells (see the Web site at www.biocytex.com, select "Product List," then "CellQuant Kits").

1.6. The Benefits of Quantitative FCM

Flow cytometers are ideally suited to examine blood cells because they can so readily measure the light scatter and fluorescence signals collected from large numbers of free-floating cells stained by specific fluorochrome conjugates. The flow cytograms—patterns of light scatter and fluorescence revealed by gating strategies—become analogous to the patterns of cellular architecture observed through the microscope. If cells are prepared in a consistent fashion and examined through a consistent window of analysis, a de facto reference frame is established by patterns from normal and abnormal cells.

With these factors in mind, we can identify several benefits of quantitative fluorescence cytometry. At the laboratory level, the most obvious benefit is the quality assurance that comes from an ongoing comprehensive evaluation of the actual signals from the flow cytometer ***(22,23)***. At the clinical level, QFCM

provides a scale that can ensure comparable staining patterns across patients, laboratories, instruments, and time—the latter being especially important when following patients through remissions and relapses. The comparability of staining patterns becomes even more important when comparing expression of markers such as CD10 that vary between normal, hyperplastic, and malignant states *(24)*.

As calibration in MESF and ABC units becomes more widespread, the clinical benefits will become more apparent. Reference ranges have been reported for the ABC values associated with many of the common surface membrane antigens expression in normal cells *(17,25)*. ABC values associated with differing levels of CD19 and/or CD20 expression in normal and malignant B cells have been used to identify distinct profiles in the various types of B-cell leukemias *(26,27)*. QFCM calibrated in ABC values was found to be more informative than conventional morphology in assessing remission status and prognosis in B-lineage acute lymphoblastic leukemia *(28)*. Changes in the ABC values for CD3 and CD7 were especially helpful in identifying and classifying malignant T cells *(25)*. QFCM may be particularly pertinent in analyzing markers of apoptosis, a critical cell function dependent on surface receptor expression *(29)*. The advent of antineoplastic therapies that target cell receptors (such as CD20, CD52, and HER2/neu) portends a much greater role for QFCM as therapies become increasingly tailored to the particular cellular characteristics of each patient *(30)*. Perhaps the most fundamental benefit of quantitative fluorescence cytometry is also the most subtle: it provides a better understanding of why staining patterns originate, how the cytometer measures them, and what cellular properties they reveal.

2. Materials

2.1. Microbead Standards

The microbead standards we employ are all obtained from Flow Cytometry Standards Corporation, San Juan, Puerto Rico 00919-4344; telephone 800-227-8143; email fcsc@caribe.net; web site www.fcstd.com. This company was, for some time, the only source of MESF-calibrated microbeads. A few other suppliers have recently begun to provide MESF calibrated microbeads, and some offer microbeads that are calibrated in "equivalent" values but do not have the environmental responsiveness or spectral matching required for true MESF assignment.

1. QC Windows kits (cat. no. 845) allow simple establishment of a common window of analysis for fluorescein and phycoerythrin conjugates. The kit includes a multilabeled reference standard, a Certified Blank microbead, and a table of initial target values for specific cytometers.

2. Quantum MESF kits (cat. nos. 823–828) include multiple levels of fluorescein- or phycoerythrin-stained microbeads in which the fluorochrome is responsive to environmental changes. Low-, medium-, and high-level kits are available.
3. Quantum ABC kits (cat. nos. 814–817) include multiple levels of microbeads coated with anti-mouse immunoglobulin to bind monoclonal conjugates. Provisional ABC values have been assigned; although they cannot be taken as exact values for any particular conjugate, they provide a general estimate of binding capacity.
4. QuickCal Software (cat. no. 350 for Macintosh and 351 for Windows) is not required to use the calibrators, but it makes data analysis easier.

2.2. Specimens

Fresh specimens are always optimal, but in practice the staining properties (including FI) of most surface markers are rather stable over at least 24 h after collection. Either heparin or ethylene diamine tetraacetic acid (EDTA) may be used as an anticoagulant; in our experience, heparin provides better light scatter patterns, especially in older specimens. Changes in the typical patterns of FI (either normal or abnormal) may suggest a specimen integrity problem.

2.3. Fluorescent Conjugates

With proper standards and characterization (*see* **Note 1**), any fluorescent conjugate suitable for FCM can be used to measure MESF and ABC values. Most systems of QFCM have used either fluorescein (often abbreviated FITC, since the isothiocyanate derivative is used to label protein) or phycoerythrin (PE). Fluorescein does not have as great a quantum yield as PE, so PE conjugates tend to be brighter and may be better for measuring fluorescence on dimly labeled cells. However, PE may also be more likely to hinder binding, since it is over twice as large as the antibody molecule.

2.4. Lysing and Fixing Reagents

If FI is to be a reliable measure of receptor binding, cell preparation and staining must not significantly alter receptor expression or conjugate binding (although some second-order effects are probably unavoidable). Most methods for hematologic applications involve lysis of red cell fixation of leukocytes before they are analyzed. Such procedures fall into one of two categories: stain-and-lyse, or lyse-and-stain. The latter method using ammonium chloride can provide good FI results in the proper hands, but the commercial stain-and-lyse procedures are probably more robust and generally give consistent results. However, at least one stain-and-lyse procedure—the Q-Prep system—should be avoided, because it diminishes staining ***(31)***. We have had good success with a saponin-based commercial lysing agent (Immuno-Lyse, Beckman-Coulter,

Hialeah, FL, www.beckman.com) and fixation with 10% methanol-free formaldehyde (Polysciences, Warrington, PA, www.polysciences.com).

2.5. Flow Cytometers

As discussed in **Subheading 1.**, virtually all clinical flow cytometers store and analyze data in the same general fashion, and any model can be calibrated for MESF with the proper standards.

3. Methods

3.1. Cytometer Calibration

Fluorescence calibration involves two sequential processes: first, adjusting PMT voltages to obtain a common window of analysis; second, analyzing MESF standards to obtain the FI calibration curve. Compensation is removed for the first adjustment, then it is applied before the MESF standards are analyzed (*see* **Note 2**). Once the instrument-specific settings for the common window of analysis have been established, a simplified daily setup may be followed. The following instructions are adapted from the product insert for QC Windows kit (*see* **Subheading 2.1.1.**) and our own procedures.

3.1.1. Establishing a Common Window of Analysis

1. Establish normal cytometer operation by manufacturer's protocol or laboratory standard procedure.
2. Set all compensation adjustments to zero (0%).
3. Dispense 2–4 drops of QC3 microbead standards from the QC Windows kit (*see* **Subheading 2.1., step 1**) into about 200 µL of the same solution in which the cellular analytes will be suspended.
4. While the QC3 microbead suspension is flowing, adjust PMT voltages such that the each peak fluorescence channel value coincides with the value for its respective fluorescence parameter listed in the the table of initial target channels provided in the package insert.
5. Adjust electronic compensation to remove any overlap in fluorescent dye emissions (*see* **Note 2**).
6. With compensation optimized, rerun the QC3 microbead standards and determine the peak channel value for each fluorescence parameter. These values become the instrument-specific-target channels.
7. Record all instrument settings and peak channels numbers (*see* **Note 3**).
8. Proceed to the MESF calibration curve.

3.1.2. MESF Calibration

1. Dispense 2–4 drops of MESF microbead standards (*see* **Subheading 2.1., step 2**) from the QC Windows kit into about 200 µL of the same solution in which the cellular analytes will be suspended.

2. Flow and acquire about 10,000 microbead events. Analyze the fluorescence of the singlet microbead population gated by light scatter. The histogram should show a blank population and four peaks with increasing FI. Determine the peak channel value (*see* **Note 3**) of each population.
3. Enter the data into the QuickCal Analysis Program or a spreadsheet program that performs linear regression. The regression should be performed with *X* values as the log of the MESF values assigned to the microbeads, and *Y* values as the peak channel numbers from the log fluorescence histogram ***(13)*** (*see* **Note 3**). The blank microbead peak channel is not included in the regression, but rather read as an unknown from the calibration curve. The slope is expressed in units of channels per decade, so the dynamic range is revealed by dividing the slope into the total number of histogram channels.

3.1.3. Daily Setup and Quality Control

1. Bring all cytometer settings (PMT voltages and compensation values) to the values obtained by adjustment under **Subheading 3.1.1.**
2. Analyze a suspension of QC3 microbead standards and determine their peak histogram channel values for each fluorescence parameter. Variance in the window of analysis is acceptable if these values lie within 1–2% of the instrument-specific target channels obtained in **Subheading 3.1.1.** If variance exceeds this range, consider further evaluation and corrective action before proceeding.
3. Insure proper compensation with the analyte of choice. Small adjustments to compensation settings may be made at this time without notably altering the window of analysis, but PMT voltages should not be changed.
4. Analyze MESF microbead standards as above to obtain the FI calibration curve and evaluate the primary performance parameters ***(13)***.

3.2. Whole Blood Staining, Lysing, and Fixing

The following preparative procedure can be adapted for high throughput using a multi-tube vortexer (VWR Scientific Products, cat. no. 58816-115, West Chester, PA 610-431-1700, www.vwrsp.com).

1. Dispense 100 µL aliquots of whole blood into each 13 × 75 polypropylene tube.
2. Add 100 µL of working conjugate solutions to their respective tubes. Vortex using a multitube vortexer for 10 s at low/moderate setting.
3. Incubate 30 min at ambient temperature.
4. Wash with phosphate-buffered saline–bovine serum albumin (PBS-BSA), then centrifuge for 3 min at 400*g* and aspirate supernatant.
5. Add 1.0 mL diluted Immuno-Lyse to each tube. Vortex two times with 2-s bursts at moderate setting.
6. Forty-five seconds after adding Immuno-Lyse, add 0.5 mL of 10% formaldehyde solution to each tube. Vortex four times with 2-s bursts at moderate setting. Add 2 mL PBS.

7. Centrifuge for 3 min at 400*g*. Aspirate supernatant. Vortex four times with 2-s bursts at moderate setting. Resuspend in PBS containing 0.2% formaldehyde. Store in refrigerator overnight.
8. Centrifuge for 3 min at 400*g*. Aspirate supernatant. Vortex four times with 2-s bursts at moderate setting. Analyze on flow cytometer.

We customarily allow preparations to fix overnight (*see* **step 7**) because light scatter patterns stabilize, but the final wash (*see* **step 8**) can be done immediately and the cells analyzed without effecting FI.

4. Notes

1. Fluorescence and binding properties of conjugates: For purposes of measuring ABC using MESF units, the effective *F/P* ratio must be known. Although the MESF of the conjugate can be determined rather simply by solution fluorimetry, fluorimetry alone cannot ensure that the antibody concentration of a conjugate solution reflects active antibody binding sites. Conjugation can denature binding sites and lead to inactive or low-affinity binding. Most conjugates are a mix of different molecular species with a range of molar *F/P* ratios, which can complicate the relationship between FI and binding properties. Specialty conjugates assessed particularly for QFCM have recently become available on a limited basis; check with your suppliers to see if they offer such products. The use of pure unimolar conjugates (all species having one fluorochrome per antibody molecule) may be worth the special preparation or purification required. The other major concern regarding conjugates is that they must essentially saturate cell receptors, which requires high affinity antibody and/or higher concentrations of conjugate than may be required for simple dichotomous phenotyping. Because concentration can vary with both source and individual lot, we recommend constructing titration curves with twofold serial dilutions on the cells of interest to insure that FI reaches the upper plateau at the concentrations employed.
2. Compensation for spectral overlap: We have not described specific methods for compensation because each laboratory must determine their own approach based on the types of specimens they receive and the approach to compensation they use. This topic is the subject of a recent comprehensive review ***(32)***, which is highly recommended. Most laboratories compensate for spectral overlap through electronic adjustments that alter FI signals; therefore, compensation must precede MESF calibration. More recently, software approaches to compensation allow unaltered FI signals to be digitized and stored; the compensation is adjusted algorithmically after acquisition. In any case, data for the MESF calibration curve standards and for stained cells should undergo the same compensation corrections, whether by hardware or software.
3. Histogram scales and values for peak channel numbers: The histogram channel number is a real physical value reflecting the binning of analog signals into channels during digitization. Because the data (in most cytometers) has been logarithmically amplified, the distribution of histogram channels reflects a logarithmic

scale, thus the channel numbers are often called "log histogram channel value" (even though the histogram channels are linear arrays). Software on cytometers often reports a theoretically linearized "mean fluorescence" or "relative fluorescence" value based on the presumption of perfect logarithmic amplification of all analog signals. Although the theoretical conversion provides a convenient log-to-linear estimate, the underlying presumption is surely false to at least some extent. We therefore prefer to use actual histogram channel values for all data regarding FI. Unfortunately, some outdated but still-in-use cytometer software fails to report the real histogram channel value, so the relative fluorescence values must be reconverted to histogram channel values ***(13)***. Some cytometers now employ direct digital acquisition of FI signals that have not been logarithmically amplified. In these systems, there is no true log histogram channel. However, the software still reports a "virtual" log histogram channel that should be used for FI calibration. A number of statistics may be used to represent the peak histogram channel; in particular, the mean, median, or modal values. The median channel is probably the single best statistic for FI calibration ***(33)***.

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4

Special Histopathology Stains

13

Methods in Leukocyte Cytochemistry

Mark G. Hanly

1. Introduction

1.1. The Basis of Leukocyte Cytochemistry

During the development and maturation of hematopoetic precursors, certain enzymes and associated substances, such as glycogen, are produced in the developing cells ***(1,2)***, which, if detected, can provide important clues to the lineage and classification of leukemias. Although the evaluation of abnormal populations of white blood cells has been greatly refined by the use of immunohistochemistry, flow-cytometric analysis ***(3)***, and molecular studies, cytochemistry still plays an important role in the assessment of the abnormal marrow and peripheral blood smear. Cytochemical studies, therefore, still have a role to play in the evaluation of leukemias and other hematological diseases *see* **Fig. 1**).

1.2. Commonly Used Cytochemical Stains

Cytochemical stains commonly used in the evaluation of hematological disorders are:

1. Myeloperoxidase stain
2. Sudan black stain
3. Periodic acid Schiff (PAS) stain
4. Naphthol AS-D acetate esterase stain (NASDA)
5. α-Naphthyl butyrate esterase stain (nonspecific esterase/NSE)
6. Combined α-naphthyl butyrate esterase and choracetate esterase stain
7. α-Naphthol acetate esterase stain (ANAE)
8. Choroacetate esterase stain (specific esterase)
9. Neutrophil alkaline phosphatase/leukocyte antigen phosphatase (NAP/LAP)

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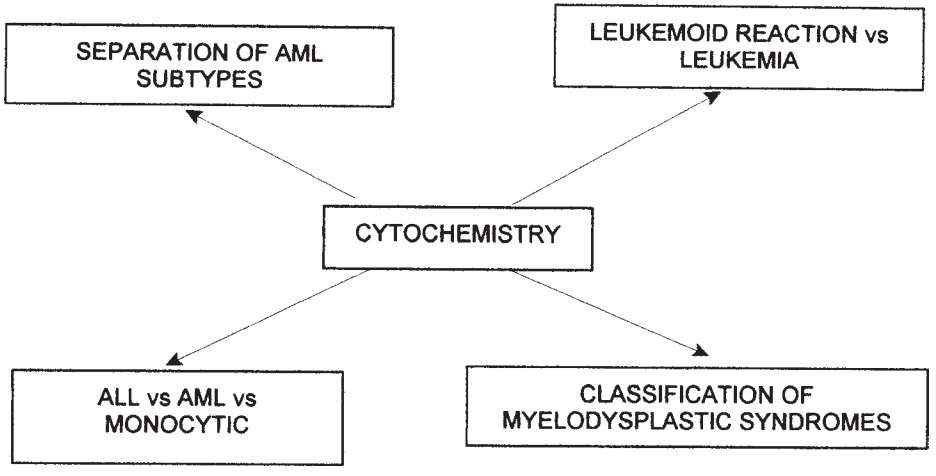

Fig. 1. Uses of cytochemical studies in the evaluation of blood and bone marrow smears.

1.3. Clinical Application of Cytochemical Stains in Hematological Disease

Cytochemical tests are of most use in the following settings:

1. Evaluation and classification of the acute leukemias with immature blast populations *(4)*
2. Differentiation of chronic myeloid leukemia (CML) from a leukemoid reaction *(5)*
3. Classification of the myelodysplastic syndromes *(6,7)*
4. Classification of chronic lymphoid leukemias (CLL) *(8)*

The use of a Sudan black or myeloperoxidase test along with choracetate esterase and ANAE studies remain important for the evaluation and classification of leukemias even if more advanced technology is available. Neutrophil (leukocyte) alkaline phosphatase is important in evaluating leukemoid reactions (*see* **Tables 1** and **2**).

2. Materials

Detailed methodology is provided as follows for the most common cytochemical studies used in the evaluation of blood and bone marrow smears. However, for the less adventurous, Sigma-Aldrich offers these tests in "kit form" that greatly simplifies the process of preparing and storing the reagents, especially in low-volume situations.

Table 1
Cytochemical Reactions Used in Differentiating the Acute Leukemias

	MP	CAE	SB	NSE	PAS
AML	Positive	Positive	Positive	M4, M5 Strong Positive	M5, M6, M7 Positive
ALL	Negative	Negative	Negative	Rare/Focal	Positive

AML, Acute myeloid leukemia; ALL, Acute lymphocytic leukemia; MP, myeloperoxidase; CAE, chloracetate esterase; SB, Sudan Black; NSE, nonspecific esterase; PAS, periodic acid-Schiff.

Table 2
The Use of Cytochemistry in the Classification of Acute Myeloid Leukemias (AML)

FAB[a]	MP[b]	SD	PAS	NASDA	NSE	ANAE	CAE
M0	–	–	–		–	–	–
M1	+	+/+++	–	+	–	–	+
M2	++	+++	–	+	–	–/+	++
M3	+++	+++	–	++	–	–/+	+++
M4	++	++	+	++	++	++	++
M5	–/+	–/+	++	+++	+++	+++	–/+
M6	–/+	–	–	++	–	+	+
M7	–	–	–	–	–	++	–

[a]French-American-British classification: M0, undiffentiated; M1, myeloid; M2, myeloid with differentiation; M3 promyelocytic; M4, myelomonocytic; M5, monocytic; M6, erythroid; M7, megakaryocytic.

[b]Stains listed in the sequence shown in the text, under **Subheading 1.2.**, but for combined esterase s stain.

2.1. Equipment

1. Coplin jars or vertical staining Jars with covers (10 of each)
2. Graduated glass pipets: 10, 25, 50, and 100 mL (2 of each)
3. Graduated narrow-mouthed Erlenmeyer flasks: 125, 250, 500, and 1000 mL (3 of each)
4. Graduated Griffin beakers: 250, 500, 1000, and 2000 mL (2 of each)
5. Graduated cylinders: 25, 50, 100, 250, and 500 mL (1 each)
6. Microscope slide coverslips
7. Microscope with oil objective lens

2.2. Reagents

Reagents required are listed under methodology for each stain (*see* **Subheading 3.**)

2.3. Specimens

Cytochemical studies can be performed on both blood and bone marrow smears and in certain circumstances they may also be utilized on marrow cores embedded in plastic resin ***(9)*** as well as in ultrastructural studies using the electron microscope ***(10,11)***. The International Committee for Standardization in Hematology (ICSH) have issued detailed recommendations for cytological procedures in hematology ***(12)***. The following fixatives are recommended for the marrow aspirate and peripheral blood smears.

2.4. Fixatives

1. Used in NASDA, and peroxidase: 10% Formal acetone. This fixative consists of 10 mL of 36% formaldehyde and 30 mL of 0.1 *M* cacodylate buffer, pH7.4 (*see* **Subheading 3.4.2., item 2**) mixed, with 60 mL of acetone.
2. Used in LAP stains: 10% Formal methanol. This fixative consists of 10 mL 36% Formaldehyde and 90 mL of absolute methanol. This fixative should be stored at 4°C.
3. Used for Sudan black B stain, α-naphthyl butyrate and chloroacetate esterase: Formalin vapor. This fixative consists of 1–2 mL of 36% formaldehyde poured into a Coplin jar.
4. Used in PAS and May-Grunwald-Giemsa stains: Absolute methanol.
5. Used in myeloperoxidase: Buffered glutaraldehyde-formaldehyde. This fixative is made by mixing 50 mL of 25% glutaraldehyde solution with 27.8 mL of 36% formaldehyde solution and making up to 1 L with 0.1 *M* phosphate buffer (pH7.3).

2.5. Buffers

1. 0.2 *M* Cacodylate Buffer
   a. Sodium cacodylate: (Na $(CH_3)_2ASO_2 3H_2O$) 34.24 gL dissolved in distilled water.
   b. Add 86.4 mL of 0.1N hydrochloric acid to above solution (final pH 7.35–7.4).

To prepare the 1 *M* buffer, dilute 1 : 1 just prior to use (7.5 mL buffer plus 7.5 mL) with distilled water.

2. Phosphate Buffer 0.1, pH 6.9
   a. Potassium dihydrogenorthophosphate (KH_2PO_4) 1.37 g/L distilled H_2O.
   b. Disodium orthophosphate (Na_2HPO_4) 14.2 g/L distilled H_2O.
   c. Mix 450 mL of **item 1** and 550 mL of **item 2** and adjust to pH 6.9.

3. Buffer Solution for Sudan Black
   a. Crystalline phenol, 16 g.
   b. Absolute ethanol, 30 mL.
   c. Distilled water, 100 mL.
   d. Disodium hydrogen phosphate, 0.3 g.

Add phenol to ethanol. Add 0.3 g of disodium hydrogen phosphate to 100 mL of distilled water. Add phenol-ethanol mixture to disodium hydrogen phosphate solution. Stir well and filter.

4. Phosphate Buffer (0.1*M*, pH 6.0) α-Naphthyl Butyrate Esterase
   a. Potassium dihydrogenorthophosphate (KH_2PO_4), 1.37 g/L distilled H_2O.
   b. Disodium orthophosphate (Na_2HPO_4) 14.20 g/L distilled H_2O.
   c. Mix 880 mL of **item 1** and 120 mL of **item 2** and adjust to pH 6.0.
5. Buffers for Combined α-Naphthyl Butyrate (Nonspecific Esterase) and Chloroacetate Esterase
   a. Sorensen's phosphate buffers pH 6.15 and pH 7.6.

3. Methods

3.1. Myeloperoxidase

3.1.1. Principle

The peroxidase in leucocytes transfers hydrogen from benzidine or other substrate to hydrogen peroxide with the production of oxidized substrate and water. The oxidized substrate is precipitated at the site of the enzyme activity as blue granules.

3.1.2. Specimen

Myeloperoxidase is not inhibited by heparin; oxalate or ethylenediaminetetraacetic acid (EDTA) and films made from such blood are suitable if made within 6 h of collection. (*See* **Note 1**.)

3.1.3. Reagents

1. Fixative: Buffered glutaraldehyde-formaldehyde.
2. Substrate: This consists of 5 mg 3′-diaminobenzidine tetrahydrochloride, 10 mL 0.05 *M* Tris-HCl buffer (pH7.6) and 0.1 mL 30% (weight/volume) hydrogen peroxide (H_2O_2). This should be prepared just before use. Add in order, mixing well between each addition.
3. Rinsing solution: Normal saline (9 g/L sodium chloride solution, Tris-HCl buffer).
4. Reaction enhancer: This consists of 0.5 g copper sulfate (Cu SO_4) dissolved in 100 mL 0.05 *M* Tris-HCl buffer (pH 7.3). (*See* **Note 2**.)
5. Counterstain: Giemsa stain.

3.1.4. Procedure

1. Fill Coplin jar 1 with fresh substrate solution.
2. Fill Coplin jar 2 with copper sulfate reaction enhancement solution.
3. Place fresh air-dried smears in glutaraldehyde/formadehyde fixative for 60 s. Rinse in saline rinse solution for 30 s.
4. Place fixed smears in Coplin jar 1 filled with substrate solution.
5. Remove smears and wash in 0.05 *M* Tris-HCl buffer, pH 7.3.
6. Place smears in Coplin jar 2 filled with copper sulfate reaction enhancement solution for 90 s.
7. Rinse in saline solution.
8. Counterstain with Giemsa stain.
9. Air-dry.
10. Mount.

3.1.5. Results

Positive myeloperoxidase activity is indicated by the presence of blue granules in the cytoplasm. The reaction fades with time; slides stored longer then 3–4 wk will lose visible granules.

3.1.6. Interpretation

3.1.6.1. Normal Cells

Myeloperoxidase is a lysosomal enzyme normally found in the azurophil granules of neutrophils and monocytes and in the specific granules of eosinophils.

1. Developing granulocytes: Always positive
2. Promyelocytes and myelocytes: Strongly positive
3. Mature neutrophils: Positive
4. Eosinophils and basophils: Positive
5. Myeloblasts: Negative
6. Promonocytes: Positive
7. Monocytes: Positive, finely granular
8. Monoblasts: Negative
9. Lymphocytes: Negative
10. Lymphoblasts: Negative
11. Plasma cells: Negative
12. Megakaryocytes: Negative
13. Erythrocytes: Negative

3.1.6.2. Leukemic Blasts

1. Acute myelogenous leukemia M1, M2, M3, M6: Strongly positivity in >5% blasts, usually more than 85% of blasts are positive.

2. Acute myelomonocytic leukemia M4: Weak-to-moderate positivity identified, granules may appear very fine.
3. Acute monocytic leukemia M5: Negative or occasional blasts showing fine "dusty" granularity.
4. Acute myelogenous leukemia M7: Negative.
5. Acute lymphocytic leukemia (ALL): Negative, but occasionally positive cells may be found; it must, by definition, be less than 5% of blasts.
6. Auer rods: Positive.

3.1.6.3. Comment

1. The main value of this stain is the distinction between acute myeloid and acute lymphoblastic leukemias as only those immature cells that show myeloperoxidase activity can be confidently referred to as myeloblasts; if the reaction is negative they could be any other type of blast cell.
2. Myeloperoxidase activity closely parallels Sudanophilia in myeloid blasts.
3. In addition, myeloperoxidase studies may be used for the evaluation of neutrophil myeloperoxidase activity in refractory anemias and cases of hereditary myeloperoxidase deficiency ***(13,14)***.

3.2. Sudan Black B

3.2.1. Principle

Sudan Black B is an inert diazo dye with an affinity for phospholipids and sterols that is insoluble in water. There is a close relationship between Sudanophilia and myeloperoxidase activity. Both reactions are positive in the azurophil granules of neutrophils and monocytes and in the specific eosinophil granules. The biochemical basis for this observation is not well undrstood, but one consideration is that the Sudan Black B stains the lipid membrane that envelop the myeloperoxidase granules in the cytoplasm of granulocytes and granulocytic precursors ***(15)***. The methodology of performing the stain has changed little since its introduction by Sheehan in 1939 ***(16,17)***.

3.2.2. Specimens

Fresh air-dried blood smears or air-dried bone marrow aspirate smears may be used. Blood collected into EDTA tubes is also acceptable. Sudanophilia is stable on dried, unfixed smears for 2–3 d.

3.2.3. Reagents

1. Fixative: Formalin vapor (*see* **Subheading 2.4.**).
2. Stain: Sudan Black B. This consists of 0.3 g of Sudan Black B dissolved in 100 mL absolute alcohol, mix the solutions well, allow the solution to stand for 12–24 h, then filter to remove particles.

3. Buffer: This consists of 16 g of crystalline phenol dissolved in 30 mL of absolute ethanol added to a mixture of 0.3 g disodium hydrogen phosphate dissolved in 100 mL of distilled water. Stir the mixture well and filter.
4. Working Sudan Black stain: This consists of 30 mL of Sudan Black B stain solution mixed with 20 mL of phenol buffer. Filter mixture and place in a dark bottle. (*See* **Note 3**.) The working solution may be kept at 4°C for up to 2 mo.
5. Differentiating solution: 70% ethanol.
6. Counterstain: May-Grünewald-Giemsa.

3.2.4. Procedure

1. Fix the air-dried smears in formalin vapor for 4 min.
2. Air-dry slides and do not rinse.
3. Incubate smears in Sudan Black working solution in a Coplin jar for 60 min.
4. Remove slides and differentiate in 70% ethanol for 2 s.
5. Rinse in distilled water immediately.
6. Air-dry slides
7. Counterstain slides using May-Grunewald-Giemsa as follows:

May-Grünewald	5 min
Giemsa	10 min
Buffer	5 min
Buffer	5 min

3.2.5. Results

Dark-brown/black punctate staining represents Sudanophilia.

3.2.6. Interpretation

Lymphocytes and blasts are negative, whereas mature and immature myeloid cells and moncytes show characteristic staining patterns.

3.2.6.1. Normal Cells

1. Developing granulocytes: Strongly Positive
2. Mature neutrophils: Strongly positive
3. Eosinophils: Strongly positive
4. Basophils: Variable
5. Myeloblasts: Positive
6. Mononcytes: Scattered fine granules or negative
7. Monoblasts: Scattered fine granules or negative
8. Lymphocytes: Negative
9. Lymphoblasts: Negative
10. Megakaryocytes: Negative
11. Platelets: Negative
12. Lipid-containing macrophages: Negative
13. Erythrocytes: Negative

3.2.6.2. Leukemic Blasts

1. Acute myelogenous leukemia M0: Negative.
2. Acute myelogenous leukemia M1, M2, M3, M6: Strongly positivity in >5% blasts, usually more than 85% of blasts are positive.
3. Acute myelomonocytic leukemia M4 : Weak to moderate positivity identified, granules may appear very fine.
4. Acute monocytic leukemia M5 : Negative or occasional blasts showing fine "dusty" granularity.
5. Acute myelogenous leukemia M7 : Negative.
6. Acute lymphocytic leukemia (ALL): Negative but occasionally positive cells may be found, must, by definition, be less than 5% of blasts.
7. Auer rods : Positive.

3.2.6.3. Comment

Sudan Black positivity virtually excludes the diagnosis of an ALL, although rare cases of Sudan Black-positive ALL have been described. ***(18)***

3.3. Periodic Acid Schiff (PAS)

3.3.1. Principle

The PAS reaction occurs as a result of the liberation of carbohydrate radicles from glycogen by periodic acid and their oxidation to aldehydes, which, in combination with the Schiff stain, produces a magenta color. A positive reaction usually denotes the presence of glycogen. The presence of glycogen can be confirmed by demonstrating that the positive reaction disappears when the smear is treated with diastase before it is stained, the staining properties of non-glycogen PAS-positive material being unchanged by the process of diastase digestion. The PAS reaction is widely used to identify intracellular and cytoplasmic carbohydrates, glycogen, mucopolysaccharides, mucoproteins, glycoproteins, and glycolipids.

3.3.2. Specimen

Fresh blood or bone marrow smears are the optimal specimen. Blood/marrow samples obtained from tubes containg EDTA are satisfactory, however.

3.3.3. Reagents

1. Fixative: Absolute methanol 50 mL placed in a Coplin jar.
2. PAS stain: This consists of 100 mL of 1% periodic acid.
3. Schiff's reagent: This is made by bringing 400 mL of distilled water to the boil and adding 2 g of pararosaniline chloride. Mix the solution and cool to 55°C and filter. Then add 40 mL of 1 *N* HCl and cool to 20°C followed by 2 g of anhydrous metabisulphite to the cooled mixture and place in the dark until straw colored

(12–36 h). At this stage add 1 g of activated charcoal and filter until the solution is colorless.
4. Counterstain: Harris' hematoxylin.
5. Enzymatic digestion solution: Dissolve 1 g of diastase powder in 1 L of saline solution.
6. Rinse: Distilled water.

3.3.4. Procedure

1. Fix air-dried slides in methanol for 10 min.
2. Remove slides from methanol and rinse in running tap water for 8 min.
3. Air-dry the slides. [control slides for diastase digestion should be exposed to diastase digestion solutuion (1 g diastase in 1 L of 9 g/1 NaCl) for 1 h at room temperature.]
4. Flood slide with periodic acid for 10 min.
5. Wash with distilled water for 30 min. in the dark.
6. Immerse slides in Schiff's reagent for 30 min.
7. Rinse stained slides in running tap water for 10 min.
8. Counterstain with Harris' hematoxylin for 10 min.
9. Rinse in tap water for 5 min.
10. Air-dry slides and coverslip.

3.3.5. Results

Glycogen stains magenta. Positivity may appear as diffuse pale pink or deep magenta, show fine or coarse granulation, or "block positivity." Diastase-digested control slides will show no or greatly reduced staining if the PAS positive material represents glycogen.

3.3.6. Interpretation

3.3.6.1. Normal Cells

1. Granulocytes: Positive at all stages of development.
2. Mature neutrophils: Intense diffuse positivity: cytoplasm contains large amounts of positive staining material in the form of granules.
3. Promyelocytes/myelocytes: Fewer granules, but cytoplasm diffusely pale pink.
4. Eosinophils: Background cytoplasm is PAS positive; eosinophil specific granules do not stain.
5. Lymphocytes: A few fine or coarse granules against a negative cytoplasmic background in 10–40% of cells.
6. Monocytes: Fine scattered granules against a pale pink cytoplasm.
7. Platelets: Strongly positive.
8. Normoblasts: Negative.

9. Red cells: Negative.
10. Myeloblasts: Negative.

3.3.6.2. Chronic Lymphoproliferative Disorders

1. B-cell CLL and B-cell PLL (prolymphocytic leukemia): Granular positivity in the majority of lymphocytes.
2. Hairy cell leukemia: Strong diffuse granular reactions.
3. Sezary cells (T cells): Weak or negative.
4. T-cell CLL: Small number of positive cells.

3.3.6.3. Acute Leukemias

1. Myeloblasts: May show diffuse or granular positivity.
2. Monocytic cells: May show block positivity.
3. Common ALL: Coarse granules or block positivity.
4. T-cell ALL: Often weakly positive.

3.3.6.4. Dyserythropoietic States

1. Erythroleukemia: Erythroblasts often positive, strong diffuse, granular, or block positivity.
2. Thalassemia: Diffuse positivity.
3. Iron deficiency anemia: Diffuse positivity.
4. Sideroblastic anemia: Diffuse positivity.
5. Myelofibrosis: Diffuse positivity.
6. Cord blood erythroblasts: Diffuse positivity.
7. Hemolytic anemia: Diffuse positivity.
8. Various types of leukemia: Diffuse positivity.
9. Aplastic anemia: Focal positivity.
10. Lead poisoning: Focal positivity.
11. Polycythemia vera: Focal positivity.
12. Pernicious anemia: positivity.

3.3.6.5. Comments

1. The PAS stain has limited value in the classification of AML with the exception of the strongly positivity noted in the erythroblasts of M6 (granular or block).
2. Positive staining is noted in AML M1–M5 to a variable extent and when present may be diffuse or finely granular.
3. Granular positivity, if identified in peroxidase/esterase negative blasts, supports a morphological diagnosis of ALL.
4. ALL L3 blasts are PAS negative.
5. PAS-positive ALL has a better prognosis than PAS-negative cases and probably represent non-B and non-T–cell proliferations.

3.4. Naphthol-AS-D Acetate Esterase Stain (NASDA)

3.4.1. Principle

Esterases are ubiquitous and form a set of enzymes that act on selected substrates. They are useful in hematology for assisting in the classification of hematological neoplasms of monocytic or myelomonocytic origin. NASDA is enzymatically hydrolized, liberating a free naphthol compound that, when coupled with a diazonium salt (fast blue BB: C17H18ClN303), forms colored deposits at the site of enzyme activity ***(20)***.

3.4.2. Specimens

Fresh air-dried blood or bone marrow smears are optimal specimens. The test may be performed on samples from tubes containing EDTA, however.

3.4.3. Reagents

1. Fixative: 10% Formal-acetone. This consists of 30 mL acetone plus 15 mL 0.1 *M* cacodylate buffer pH 7.4 (7.5 mL 0.2 *M* cacodylate buffer added to 7.5 mL of distilled water) mixed with 5 mL 35% formaldehyde.
2. 0.2 *M* Cacodylate buffer: This consists of 34.24 g/L of sodium cacodylate (Na $(CH_3)_2ASO_2 \cdot 3H_2O$) dissolved in distilled water to which 86.4 mL of 0.1 *N* hydrochloric acid is added for a final pH of 7.35–7.4.
3. Phosphate buffer 0.1 *M*: pH 6.9, which consists of 1.369 g/L solution of potassium dihydrogenorthophosphate (KH_2PO_4) mixed with a 14.196-g/L solution of disodium orthophosphate (Na_2HPO_4) mixed in a 45:55 ratio, with the pH adjusted to 6.9.
4. NASDA substrate solution: Consists of 80 mL of NASDA added to 15 mL acetone. (*See* **Note 4**.)
5. Counterstain: 0.1% Safranin.
6. Incubation mixture: This consists of 76 mL of 0.1 *M* phosphate buffer to which is added, in sequence, 2 mL propylene glycol, 1.6 mL NASDA substrate solution, and 100 mg of fast blue BB salts. The pH is adjusted to 6.9 and the solution filtered into a 100-mL Coplin jar.
7. Sodium fluoride inhibition solution: Add 90 mg sodium fluoride to 100 mL of the incubation mixture. Allow the sodium fluoride to dissolve. Carry out a simultaneous test using the inhibitory solution. The sodium fluoride solution will specifically inhibit the NASDA reaction in monocytes.

3.4.4. Procedure

1. Fix smears in formal-acetone for 30 s.
2. Rinse smears immediately in distilled water three times.
3. Air-dry the smears.

4. Prepare the incubation mixture—one with and one without the sodium fluoride inhibition mixture.
5. Filter the incubation mixture into two Coplin jars marked "plain" and "NaF."
6. Incubate both sets of slides at room temperature for 70 min.
7. Rinse both sets of smears in distilled water.
8. Counterstain for 5 min.
9. Rinse in distilled water.
10. Air-dry smears.

3.4.5. Results

Nonspecific esterase activity appears as blue granules in the nucleus and cytoplasm of both the monocytic and granulocytic series.

3.4.6. Interpretation

3.4.6.1. Normal Cells

1. Monocytes: Strong diffuse positivity.
2. Granulocytes: Weaker granular staining pattern without the addition of sodium fluoride.

3.4.6.2. Leukemic Blasts

Monocytic/myelomonocytic blasts: Positive staining.

3.4.6.3. Comment

In smears that have been exposed to a sodium fluoride "inhibition solution," the granulocyte remain positive, but the reaction cells of the monocytic blast are markedly or completely inhibited. (*See* **Note 5**.)

3.5. α-Naphthyl Butyrate Esterase

3.5.1. Principle

α-Naphthyl butyrate is the substrate in this reaction. With hydrolysis, α-naphthyl is liberated and then coupled with hexazotized pararosaniline to produce an insoluble, red-brown chromogen ***(21)***.

3.5.2. Specimens

Air-dried blood or bone marrow smears fixed in formalin vapor.

3.5.3. Reagents

1. Fixative: Formalin vapor (*see* **Subheading 2.4.**).
2. Phosphate buffer (0.1 *M* pH 6.0): This consists of a 1.37-g/L solution of potassium dihydrogenorthophosphate (KH_2PO_4) mixed with a 14.2-g/L solution

of disodium orthophosphate (Na_2HPO_4) mixed in a ratio of 88:12 with the pH adjusted to 6.
3. α-Naphthyl butyrate substrate: This consists of 0.2 mL of α-naphthyl butyrate added to 10 mL of N,N-dimethyl formamide. This solution should be stored at –20°C.
4. 4% Pararosaniline solution: This consists of 1 g pararosaniline HCl dissolved in 25 mL warm 2 *N* hydrochloric acid: This mixture should be filtered before storing at 4°C.
5. 4% Sodium nitrite solution: This consists of 1 g of sodium nitrite ($NaNO_2$) dissolved in 25 mL distilled water. This solution should be made fresh for every test.
6. Hexazotized paraosaniline: This consists of equal volumes of 4% pararosaniline and sodium nitrite solution added together.
7. Counterstain: 0.2% methyl green.

3.5.4. Procedure

1. Fix slides in formalin vapor for 4 min.
2. Dry the back and sides of the slide carefully.
3. Prepare incubation mixture as follows: Add 0.2 mL pararosaniline (do not splash sides of container) to 0.2 mL sodium nitrite and simultaneously start the timer. Mix together for exactly 3 min then add 76 mL 0.1 *M* phosphate buffer, pH 6.0, and 4 mL α-naphthyl butyrate substrate and filter into a Coplin jar.
4. Place test smears in the incubation mixture.
5. Incubate slides at room temperature for 45 min.
6. Rinse once with distilled water.
7. Counterstain with methyl green for 2–4 min.
8. Rinse with distilled H_2O.
9. Air-dry.

3.5.5. Results

The positive reaction appears as a red-brown color.

3.5.6. Interpretation

3.5.6.1. Normal Cells

1. Myeloblasts	0 – +
2. Promyelocytes	0 – +
3. Neutrophils	0 – +
4. Basophils	0
5. Eosinophils	0 – +
6. Monocytes	+++
7. Lymphocytes	0 – +
8. Plasma Cells	++

9.	Platelets	+++
10.	Megakaryocytes	+++
11.	Reticulum cells	+++

Key: 0, Negative; +, weak (not significant); ++ moderate; +++ strong.

3.5.6.2. Leukemic Blasts

1. T-cell ALL: Localized positivity.
2. AML: Negative.
3. ALL: Usually negative or weak with scattered granules in null-cell ALL.
4. AMML (acute myelomonocytic leukemia): Positive in monocytic lineage.
5. Hairy cell leukemia: Crescentic positive staining.
6. Myeloma. Strongly positive.
7. Megaloblasts: Positive.
8. Erythroblasts in M6 AML: Positive.
9. B-CLL: In most cases, the lymphocytes are negative as expected in B lymphocytes. A small proportion of cases show scattered granules or a crescentic distribution similar to that of hairy cell leukemia ***(21,22)***.
10. T-CLL, T-PLL, Sezary cells: Characteristic localized activity in a proportion of cells.
11. Gaucher's disease: Positive.

3.5.6.3. Comments

1. α-NBE is a strong monocyte marker that is superior to NASDA or ANAE.
2. Positive stain facilitates the diagnosis of AML M5, in which the cells will exhibit a very strong diffuse staining reaction.
3. α-NBE may demonstrate exhibit fluoride-resistant localized activity in a proportion of normal T lymphocytes with a typical pattern of dot positivity in T-helper cells.
4. α-NBE reveals a characteristic pattern of activity in hairy cell leukemia with fine, scattered granularity associated with a coarser granulariy in a crescentic distribution. The activity is sodium fluoride resistant.
5. α-NBE may be used in combination with chloracetate esterase in a single method: the so-called combined or dual esterase reaction, which demonstrates both types of esterase in a single preparation. This simplifies the cytochemical characterization of leukemia cells, and, in cases of AML M4, it helps to identify both the granulocytic and monocytic components (*see* **Subheading 3.6.**).

3.6. Combined α-Naphthyl Butyrate and Chloroacetate Esterase

3.6.1. Principle

This stain is used to examine myeloid and monocytic cells on one slide. It is useful in the diagnosis of AML M4.

3.6.2. Specimens

Air-dried blood or bone marrow smears fixed in formalin vapor.

3.6.3. Reagents

1. Fixative: Formalin vapor.
2. α-Naphthyl butyrate stock solution as described in **Subheading 3.5.3.**
3. Pararosaniline working solution: This consists of 1.0 g pararosaniline dissolved in 25 mL of 2 *N* hydrochloric Acid.
4. 4% Sodium nitrite solution as described in **Subheading 3.5.3.**
5. Buffers: Sorensen's Phosphate buffers, pH 6.15 and pH 7.6.
6. α-NBE working solution: This consists of 0.2 mL pararosaniline mixed with 0.2 mL 4% sodium nitrite. The solution is allowed to react for 3 min. Then, 76 mL of Sorensen's phosphate buffer (pH 6.15) is added along with 4 mL of NBE stock solution. The pH is adjusted to 6.16 and the solution is filtered.
7. Sodium fluoride monocyte inhibition solution: Add 15 mg sodium fluoride to 80 mL of the α-naphthyl butyrate working solution and filter.
8. Chloracetate esterase working solution: Dissolve 0.1 g naphthol AS-D chloroacetate in 0.5 mL N,N-dimethyl formamide. Dissolve 0.005 g fast blue BB salt in 9.5 mL of Sorensen's phosphate buffer, pH 7.6. Mix both solutions together and filter before use.
9. Counterstain: Harris's hematoxylin.

3.6.4. Procedure

1. Fix slides in formal vapor for 4 min.
2. Clean the sides and back of the slide with a tissue.
3. Incubate in α-NBE working solution at room temperature for 45 min.
4. Rinse with distilled water.
5. Incubate in chloroacetate esterase working solution for 10 min.
6. Rinse with distilled water.
7. Counterstain: Place slides in Harris's hematoxylin for 5 min, then rinse again in running water for 10 min.
8. Air dry slides.

3.6.5. Results

Positive staining is indicated by either a red-brown coloration or a dark blue coloration

3.6.6. Interpretation

Monococytic cells (normal and neoplastic) are stained red-brown, whereas myeloid cells (normal and neoplastic) are stained dark blue. The capacity of distinguishing cells with a myeloid lineage from those with monocytic lineage

on a single slide makes this stain especially useful in the evaluation of AML with monocytoid features.

3.7. α-Naphthol Acetate Esterase (ANAE) (22)

3.7.1. Principle

α-Naphthyl acetate is hydrolyzed by enzymes in leukocytes, liberating a free naphthol compound that, when coupled with a diazonium salt, forms highly colored deposits at sites of enzyme activity.

3.7.2. Specimens

Fresh air-dried peripheral blood or bone marrow aspirate smears.

3.7.3. Reagents

1. Fixative: Phosphate-buffered acetone formaldehyde: This is made by adding 40 mL of acetone to 25 mL 35% formaldehyde followed by 20 mL sodium hydrogen phosphate (Na_2HPO_4), 100 mL potassium phosphate (KH_2PO_4), and 30 mL distilled water. The solution must be filtered before use.
2. Substrate/incubation solutions: This consists of 100 mg α-naphthyl acetate dissolved in 5 mL methanol and added to 89 mL phosphate buffer (0.1 *M*, pH7.6). To this solution add 3 mL hexazotized pararosaniline (3 mL pararosaniline mixed with 3 mL 4% sodium nitrite); adjust the pH to 6.1 with 1 *M* sodium hydroxide.
3. Counterstain: methyl green.

3.7.4. Procedure

1. Fix stains in acetone-formaldehyde for 1 min.
2. Rinse slides in running water for 2 min.
3. Incubate slides in incubation mixture for 45 min.
4. Wash in running water for 1 min.
5. Mount while wet.

3.7.5. Results

Positivity is shown by reddish-brown staining with the cell nuclei staining green.

3.7.6. Interpretation

ANAE gives distinct patterns in lymphocytes (a dotlike reaction) and in monocytes (a diffuse positive reaction). The localized reaction in lymphocytes is resistant to the addition of sodium fluoride (NaF), whereas the reaction in monocytes is sensitive. The ANAE cytochemical reaction, as applied to

the study of leukemias and lymphoproliferative disorders, has three main applications:

1. In AML it facilitates the diagnosis of monocytic leukemia (FAB M5), the cells of which give a strong diffuse reaction sensitive to NaF. In erythroleukemia (FAB M6) and megakaryoblastic leukemia (FAB M7) the blast cells give a positive ANAE reaction localized to the Golgi zone and sensitive to NaF. In contrast to M5, the reaction in M6 and M7 is not observed when α-naphtyl butyrate is used as a substrate.
2. In ALL, ANAE, in conjunction with the acid phosphatase reaction, helps to identify T-cell-ALL.
3. In the chronic B- and T-lymphoid leukemias ANAE helps to distinguish T-cell-PLL (positive reaction) from B-cell-PLL (negative reaction) ***(24)***.

3.8. Chloroacetate Esterase Stain (Specific Esterase)

3.8.1. Principle

Naphthol AS-D chloroacetate is used as the substrate. After hydrolysis, the liberated naphthol couples with hexazotized pararosaniline to form bright red granules at the site of enzyme activity.

3.8.2. Specimens

EDTA samples are satisfactory. (*See* **Note 6.***)*

3.8.3. Reagents

1. Fixative: Formalin vapor (*see* **Subheading 2.4.**).
2. Pararosaniline working solution: This consists of 1.0 mL pararosaniline added to 25 mL 2 *N* HCl.
3. 4% Sodium nitrite solution: This is made by adding 0.4 g sodium nitrite to 10 mL distilled water and mixing well.
4. Chloroacetate esterase working solution: Dissolve 0.1 g naphthol AS-D chloroacetate to 0.5 mL N,N-dimethy formamide then dissolve 0.005 g fast blue BB salt in 9.5 mL Sorensen's phosphate buffer, pH 7.6. Mix both solutions together and filter before use.
5. Counterstain: 2% methyl green.

3.8.4. Procedure

1. Fix air-dried slides in Formal vapor.
2. Clean the back and sides of the slides with a tissue.
3. Place slides in chloroacetate working solution for 10 min.
4. Rinse in distilled water.

5. Counterstain in 2% methyl green for 2 min.
6. Rinse briefly with distilled water.
7. Air-dry.

3.8.5. Results

Enzymatic activity is indicated by bright red granules in the cytoplasm. The reaction is confined mainly to the granulocyte series and mast cells.

3.8.6. Interpretation

Chloroesterase activity develops during granulocyte maturation and appears at a later stage than myeloperoxidase activity. The reaction roughly parallels that of Sudan Black and peroxidase, but may be absent in undifferentiated myeloblasts that are peroxidase positive and are therefore of less value than peroxidase in differentiating M1 and M2 from L1 or L2. This reaction helps in differentiating the myeloblastic and monocytic cell lines. Together with α-naphthyl butyrate it helps in identifying M4 (*see* **Subheading 3.6.**) Strong activity has been reported in the abnormal erythroid precursors of Di Guglielmo's disease.

3.8.6.1. Normal Cells

1. Myeloblasts: V
2. Promyelocytes: +++
3. Neutrophils: +++
4. Basophils: 0 – +
5. Eosinophils: 0
6. Monocytes: 0 – +
7. Lymphocytes: 0
8. Plasma cells: 0
9. Mast cells: +++
10. Platelets: 0
11. Reticulum cells: 0 – +
12. Megakaryocytes: 0 – +

3.8.6.2. Leukemic Blasts and Other Hematological Diseases

1. AML (M1 and M2)	– ++/+++
2. Auer rods	– positive
3. M3	– +++
4. AMML	– V/ + or ++
5. AMOL (acute monocytic leukemia)	– 0 – +
6. ALL	– 0
7. Di Guglielmo's disease	– +++ in abnormal erythroblasts

Key: 0, negative; +, weak, not significant; ++, moderate; +++, strong; V, variable, usually negative but occasionally shows weak-to-moderate activity.

3.9. Neutrophil/Leukocyte Alkaline Phosphatase (NAP/LAP)

3.9.1. Principle

Alkaline phosphate hydrolyses naphthol AS-BI phosphoric acid at pH 9.3, and the liberated naphthol is coupled with fast blue BB to form an insoluble blue precipitate, which demonstrates the presence of enzymatic activity in fixed blood or bone marrow films ***(25)***.

3.9.2. Samples

Fresh air-dried smears are preferred. If anticoagulated blood has to be used, heparin is the preferred anticoagulant.

3.9.3. Reagents

1. Fixative: 10% Formal-methanol (*see* **Subheading 2.4.**).
2. Buffer: 0.2 *M* Tris-HCl, pH 9.3, obtained by mixing 250 mL 24.23 g/L Tris-(hydroxymethyl)aminomethane with 5 mL 1 *N* HCl and making up to 1 L with distilled H_2O.
3. Substrate solution: This consists of 10 mg naphthol AS-BI phosphoric acid sodium salt dissolved in 40 mL of 0.2 *M* Tris-HCl buffer.
4. Coupler: 30 mg fast blue.
5. Counterstain: 0.1% Safranin.

3.9.4 Procedure (see **Note 8**.)

1. Select two smears from each patient.
2. Fix air-dried smears without delay in cold fixative (0–4°C) for 30 s.
3. Rinse in distilled H_2O three times. Air-dry.
4. Prepare substrate solution by adding 10 mg of naphthol AS-BI phosphoric acid to 40 mL of 0.2 *M* Tris-HCl (pH 9.3), allow the mixture to warm to room temperature, then add 30 mg of fast blue BB. Filter solution into a Coplin jar.
5. Place slides in the Coplin jar, cover, and incubate at room temperature for 30 min.
6. Rinse well in distilled water.
7. Counterstain with Safranin for 2 min.
8. Rinse very rapidly with distilled H_2O.
9. Air-dry.

If performed at pH5 this procedure may be used for the evaluation of acid phosphatase activity. Acid phosphatase activity is detected in most hematopoetic cells. However, if the acid phosphatase activity is tested for "tartrate resistance" by the addition of crystalline tartaric acid to the working solu-

tion, a positive cytochemical reaction indicates the presence of acid phosphatase isoenzyme 5. Reactivity for this isoenzyme is key to the diagnosis of hairy cell leukemia ***(26)***

3.9.5. Results

Alkaline phosphatase activity is indicated by bright blue granules. The cell nuclei are stained red. Activity in the blood is virtually confined to bands and segmented neutrophils.

3.9.6. Interpretation

Monocytes, lymphocytes, erythroblasts, platelet, basophils, and usually eosinophils are negative. In normal bone marrow, positivity is seen in reticuloendothelial cells and osteoblasts. As alkaline phosphatase is a heat-labile enzyme, it is essential to avoid any high temperature until the reaction is complete.

3.9.6.1. Scoring NAP/LAP Activity

Based on the intensity of staining and the number of blue granules in the cytoplasm of the neutrophils, individual cells can be rated as follows:

0: negative, no granules
1: faint diffuse positivity with very few granules
2: diffuse positivity with moderate number of granules
3: strong positivity with numerous granules
4: very strong positivity with cytoplasm crowded with granules

1. The score in an individual film consists of the sum of the scores of 100 consecutive neutrophils or band forms, giving a possible total of 0–400.
2. Earlier precursors of neutrophils must not be included in the count.
3. Cells for scoring are selected from the thin portion of the smear where the red cells barely touch one another.
4. A source of error is inadequate counterstaining and failure to identify and include neutrophils with no activity.
5. The normal range is 35–100. As the scoring technique is subjective, each laboratory should determine its own normal range with its techniques, reagents, and scoring criteria.
6. The score is higher in women and children than in men (newborn infants have 150–300 neutrophils).

The major use of NAP is in the study of chronic granulocytic leukemia and the non-leukemic myeloproliferative disorders.

3.9.6.2. Conditions Associated with Low Scores

1. Untreated CML (Philadelphia chromosome positive)
2. CML in relapse

3. AML
4. Paroxysmal nocturnal hemoglobinuria

In CML, the low initial scores may change to normal or high in about one third of patients during remission; LAP scores become high during severe infections, after splenectomy, and in up to 50% of cases undergoing blast-cell transformation.

3.9.6.3. Conditions Associated with High Scores

1. Neutrophilia of infection
2. Pregnancy
3. Leukemoid reactions
4. Cirrhosis
5. Down's syndrome
6. Polycythemia rubra vera
7. Essential thrombocythemia/myelofibrosis
8. Active Hodgkin's disease
9. Aplastic anemia
10. CML—blast-cell transformation
11. Corticosteroid treatment

3.9.6.4. Conditions Associated with Normal Scores

1. Lymphocytic leukemia
2. Secondary polycythemia due to stress, hypoxia, and renal disease

3.9.6.5. Conditions Associated with Intermediate Scores

1. Monocytic leukemia
2. Myelomonocytic leukemia

4. Notes

1. Films should then be fixed and may be kept for up to 1 wk at 4°C. films made from fresh blood may be stored up to 2 wk in the dark at room temperature.
2. This must be made fresh when needed.
3. The working solution may be kept at 4°C for up to 2 mo.
4. This must be stored in a dark glass container at 4°C.
5. Monocytes sometimes retain a small degree of activity, and the two slides must be compared. Positive staining indicates monocytic or myelomonocytic differentiation. If the smears are not inhibited by the addition of sodium fluoride, this indicates granulocytic differentiation.
6. Air-dried smears can be kept for many years without loss of enzyme activity. This is a great advantage, as the method can be used on archived slides without regard to any special precautiuons in storing the smears.
7. EDTA has an inhibitory effect on LAP activity. If only EDTA blood is available, however, films should be made within 30 min of collection. The smears must be fixed without delay. If staining is to be delayed for more than about 5 h, store

the fixed films at –20°C. Fixed films may be stored at this temperature for at least 6 wk. This permits the use of stored films for quality control. If a patient's white cell count is above 5.0 × 10%/L (40–50% neutrophils) unprocessed peripheral blood smears are acceptable, but if the patient's white cell count is below 5.0 × 10%/L, obtain 5 cm^3 of blood in EDTA and **immediately** make buffy-coat smears. Controls for this test should include films, previously fixed and stored, of a known score. Negative controls are obtained by using previously fixed smears that had been inactivated by immersion in boiling water for 1 min or from an EDTA blood sample stored for 24 h. Positive controls can be obtained from a woman in the third trimester for pregnancy or from a patient with a polymorphonuclear leucocytosis due to an infection. Control slides, already fixed, can be stored in the deep freeze for ± 6 wk.

8. As the stain tends to fade fast, slides should be examined within a few hours of preparation. Some immersion oils also cause fading.

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14

Immunohistochemistry in Leukemias and Lymphomas

Marina Jaramillo, Catherine Rangel, and Thomas Grogan

1. Introduction

1.1. Principles of Immunohistochemistry

The characterization of the antigenic and immunologic properties of lymphoid and hematologic neoplasms has dramatically increased our understanding of the biology of these diseases and at the same time greatly enhanced our diagnostic abilities. Immunophenotyping has helped delineate specific lineage aberrancies indicative of malignant transformation, such as monoclonality, in the form of light- and/or heavy-chain immunoglobulin restriction, or loss of pan-B or pan-T cell antigens, or aberrant crosslineage coexpression of antigens. Unique immunophenotypic profiles have now been described for most forms of lymphoma and leukemia. Classic Hodgkin's disease, for example, has the singular combination of CD15 and CD30 positivity, in the absence of CD45 (*see* **Fig. 1**). Additional examples are mantle cell lymphoma with its unique nuclear expression of cyclin D1, and hairy cell leukemia, characterized by coexpression of CD22 and CD11c, a monocytoid marker. Beyond its diagnostic utility, immunohistochemistry (IHC) may have prognostic significance reflecting the biologic behavior of these diseases; a case in point is CD10 in large B-cell lymphomas, whose absence conveys a worse prognosis.

The introduction of new specialized techniques, such as immunocytochemistry and immunohistochemistry, the use of a growing number of highly specific monoclonal antibodies (*see* **Table 1**), the introduction of antigen-retrieval techniques, and the recent development of automated instruments have further enhanced our diagnostic accuracy, and significantly decreased time to diagnosis. Furthermore, the advent of automated IHC has multiplied the number of

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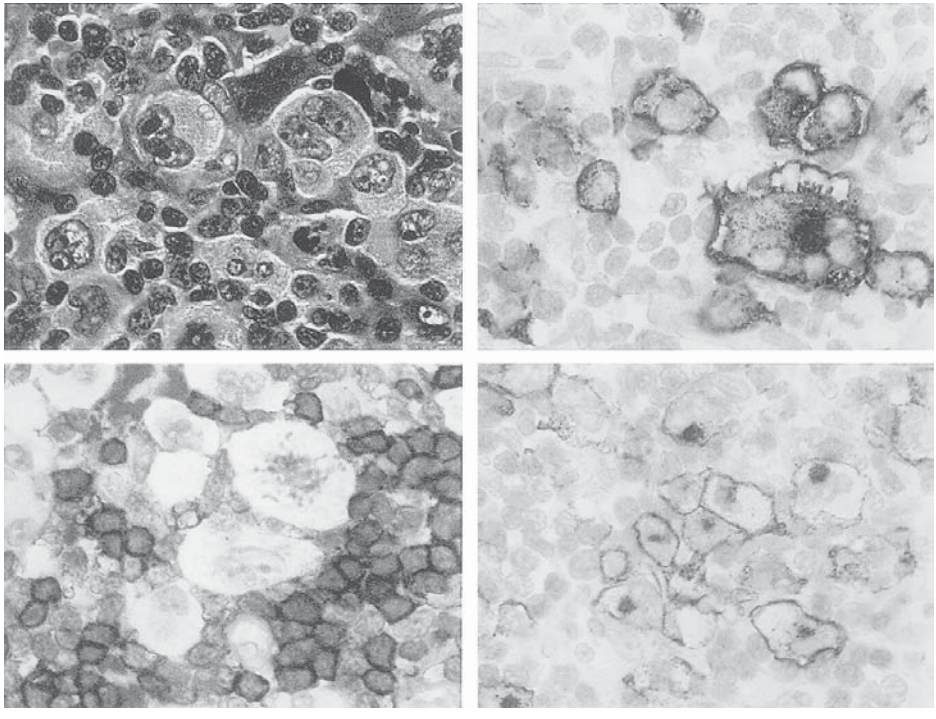

Fig. 1. Classic Hodgkin's disease phenotype. Reed-Sternberg cell (upper left) coexpressing CD15 (upper right) and CD30 (lower right), in the absence of CD45 (lower left).

Table 1
Key Human Leukocyte Antigens

CD	Other names	Cell type	Function
CD1a	T6, Leu 6	Thy, LC, DC	T-cell response regulation
CD2	T11, Leu 2	Thy, T, NK	CD58 Rp
CD3	T3, Leu 4	T, Thy, NK	TCR associated
CD4	T4, Leu 3	Thy, h/iT, PB-M	MHC Class II; HIV Rp
CD5	T1, Leu 1	Thy, T, B subset	CD72 ligand; B-cell Rp
CD7	Leu 9	Thy, T subset, NK	Unknown
CD8	T8, Leu 2	Thy, s/cT, NK sub	MHC Class I Rp
CD10	CALLA	Pre-B, GC, Neu, some Epi	Membrane-associated peptidase
CD11a	LFA-1α?chain	Leu	ICAM ligand
CD11b	Mo1, Mac-1α	G, M, NK, T/B subsets	C3bi Rp
CD11c	Leu M5, p150/95, CR4	M, NK, G, T/B subsets, HCL	C3bi Rp; CD18
CD13	gp150, aminopeptidase N	Mye progenitors, End, Epi	Unknown
CD15	Leu M1, X-hapten	G, M, LC, some Epi, RSC	Unknown
CD16	Leu 11, FcγRIII	NK, G, M	FCγ receptor III

Table 1 *(continued)*

CD	Other names	Cell type	Function
CD18	LFA-1 β chain	Leu	β chain to CD11
CD19	B4, Leu 12	B, DRC	CD21 ligand
CD20	B1, Leu 16, Leu 26	B	Ca ion channel
CD21	B2, CR2	B subset, DRC, MZ	C3d Rp; EBV Rp
CD22	BL-CAM, Leu 14	B cell, HCL	CD45RO ligand
CD23	BLAST-2, FcεRII	B subset, M, DRC, LC	Fc receptor II
CD25	Tac, IL-2 Rp	Act T/ B/M, HCL	IL-2 Rp α chain
CD28	Tp44, T44	Thy, T subset, PC	B7 (CD80) ligand
CD30	Ki-1, Ber-H2	Act T/B/NK; RSC, ALCL	CD 153 ligand
CD33	p67	Mye progenitors, M, G	Unknown
CD34	My10, GP105-120	Progenitors, End	Cell-cell adhesion
CD38	T10	Plasma cell	ADP ribosyl cyclase
CD43	Leukosialin, sialophorin	Leu (except B), PC subset	CD54 ligand
CD44	Pgp-1, H-CAM	Broad expression	Homing receptors
CD45	T200, LCA	Leu	Tyrosine phosphatase
CD45RO	UCHL-1	T, B subset, M	CD22 ligand
CD45RA	4KB5	B, T subset, M	Unknown
CD54	ICAM-1	Broad activation	LFA-1, Mac-1 ligand
CD56	NCAM, NKH1, Leu 19	NK, T subset, MM, CNS	Isoform of N-CAM
CD57	Leu 7, HNK1	NK, T/B subsets	Unknown
CD68	gp110	M	Unknown
CD77	Gb3, BLA	GC, Burkitt's, FL	CD19 ligand
CD80	B7, BB1	Act T/B, M	CD28, CD152 ligand
CD83	HB15	LC, DC	Fascin
CD86	B70, B7-2	IDC, LC	CD28, CD152 ligand
CD99	E2, MIC2	hematologic cells, ES	T-cell adhesion, apoptosis
CD43	Leukosialin, sialophorin	Leu (except B), PC subset	CD54 ligand
CD44	Pgp-1, H-CAM	Broad expression	Homing receptors
CD45	T200, LCA	Leu	Tyrosine phosphatase
CD45RO	UCHL-1	T, B subset, M	CD22 ligand
CD45RA	4KB5	B, T subset, M	Unknown
CD54	ICAM-1	Broad activation	LFA-1, Mac-1 ligand
CD56	NCAM, NKH1, Leu 19	NK, T subset, MM, CNS	Isoform of N-CAM
CD57	Leu 7, HNK1	NK, T/B subsets	Unknown
CD68	gp110	M	Unknown
CD77	Gb3, BLA	GC, Burkitt's, FL	CD19 ligand
CD80	B7, BB1	Act T/B, M	CD28, CD152 ligand
CD83	HB15	LC, DC	Fascin
CD86	B70, B7-2	IDC, LC	CD28, CD152 ligand
CD99	E2, MIC2	Hematologic cells, ES	T-cell adhesion, apoptosis

B, B-cells; PC, plasma cells; T, T-cells; h/iT, helper/inducer T; s/cT, suppressor/cytoctoxic T; Thy, thymocytes; GC, germinal center B-cells; MZ, mantle cells; G, granulocytes; Leu, leukocytes; M, monocytes/macrophages; Mye, myeloid; NK, natural killer; PL, platelets; Eo, eosinophils; Ery, red cells; DC, dendritic cells; IDC, interdigitating dendritic cells; LC, Langerhans' cell; End, endothelial cells; Epi, epithelial cells; RSC, Reed Sternberg cells; ALCL, anaplastic large cell lymhoma; FL, follicular lymphoma; HCL, hairy cells leukemia; MM, multiple myeloma; ES, Ewing sarcoma; CNS, central nervous system; Rp, receptor; FN, fibronectin; Act, activated.

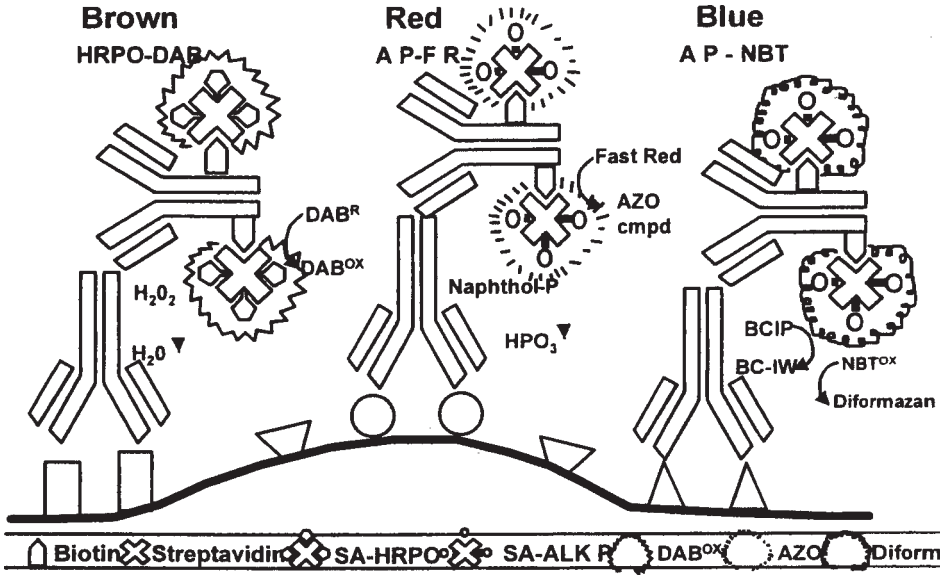

Fig. 2. Diagrammatic representation of multichromogen immunohistochemical detection system that uses biotin-streptavidin and streptavidin enzymes links to react with chromogens. HRPO, horseradish peroxidase; DAB, diaminobenzidine; AP, alkaline phosphatase; FR, fast red; AZO, diazonium compound; NBT, nitroblue tetrazolium; BCIP, 5-chromo-4-chloro-3-indolyl phosphate; BC-IW, BC-indigo white; SA, streptavidin.

assays that can be performed in a given time and the number of patients that can be tested simultaneously. These techniques can be applied to both solid tissues (lymph nodes, spleen) and cell suspensions (bone marrow aspirate, blood, and body fluids), the latter being particularly relevant in the study of leukemias. Originally, most antibodies required frozen tissue; more recently, an increasing number of antibodies can be applied to formalin-fixed, paraffin-embedded tissue, which is the universally applied method of tissue preservation. The latter development has opened the door to the testing of archival material, both for research and diagnostic purposes. As depicted in **Fig. 2**, we use a four-step multichromogen detection system ***(1)***. This method can be implemented manually or with the aid of an automated instrument and allows rapid, sensitive, and simultaneous detection of several antigens in multiple colors. The four steps involved in this system include the following.

1.1.1. Application of the Primary Antibody

The primary antibody is either a monoclonal mouse antihuman hybridoma antibody or a polyclonal heteroserum antihuman rabbit or goat antibody. Mono-

clonal antibodies are produced by clones of plasma cells, and are antigenically identical within a given clone. They are directed to a specific epitope, and are highly specific for the antigen against which they were produced. Different plasma cell clones produce polyclonal antibodies, and, as a result, these antibodies are antigenically diverse and react with different epitopes of the antigen against which they were generated. This explains the lower specificity and the more frequent occurrence of cross reactivity with other antigens.

1.1.2. Cocktail of Secondary Biotinylated (Biotin-Labeled) Antibodies

This universal cocktail includes two goat anti-mouse antibodies, IgG and IgM, and a goat anti-rabbit IgG. This combination of secondary antibodies ensures the detection of a wide spectrum of both monoclonal and polyclonal primary antibodies. The purpose behind the use of secondary antibodies is to increase the sensitivity of the assay and enhance the intensity of the signal. The attached vitamin B (biotin) serves as a critical chemical link to the enzyme-chromogen detection to follow.

1.1.3. Streptavidin Linked to an Enzyme

Streptavidin is a large molecule that binds tightly to biotin, thus ensuring permanent binding to the secondary antibody without diffusion or loss of the immunohistochemical signal. The biotin-bound streptavidin is bound in turn to an enzyme complex with either horseradish peroxidase (HRP) or alkaline phosphatase. In the presence of hydrogen peroxide (H_2O_2), horseradish peroxidase is capable of reacting with a variety of chromogens to produce a colored precipitate. The main problem associated with the use of this enzyme is the necessity to block the endogenous peroxidase naturally occurring in red cells, macrophages, and neutrophils. An alkaline phosphatase removes and transfers phosphate groups from organic esters. Although not widely used, alkaline phosphatase has the advantage of not been interfered by endogenous peroxidase. Consequently, this technique is particularly useful for immunostaining specimens with a high content of endogenous peroxidase, such as bone marrow and blood.

1.1.4. Chromogen

A chromogen is a colorless substance that becomes colorized when oxidized or reduced by another compound or enzyme. The linked enzyme plays the role of the oxidizing or reducing substance within this particular system. The resultant colored precipitate (dye) marks the site where the antigen–antibody complex is located. Different chromogens can be used to obtain different colors. Diaminobenzidine-horseradish peroxidase (DAB-HRPO) is used to produce a brown precipitate; the H_2O_2 serves as the initial oxidizing sub-

stance acted upon by HRPO. To generate a red precipitate, naphthol serves as the substrate for the enzyme alkaline phosphatase, which reacts with the fast red chromogen to produce a stable red azo compound. To produce a blue precipitate, alkaline phosphatase acting on the 5-bromo-4-chloro-3-indolylphosphate (BCIP) substrate reduces the nitroblue tetrazolium (NBT) chromogen. Finally, counterstaining with hematoxylin or methyl green adds morphologic detail, facilitating the interpretation of the immunophenotypic results.

1.2. Automated Immunohistochemistry

In our laboratory, we use a kinetically driven, automated IHC device ***(1)***. This instrument mixes and heats the different reagents, accelerating the chemical reactions with rapid achievement of the point of equilibrium. In doing so, the automated stainer dramatically reduces the time required for the completion of the assay, and ensures more consistent and clinically timely results. Machine-driven mixing ensures a uniform and speedy antigen–antibody interaction. In particular, manual IHC proceeds slowly via diffusion based on simple Brownian motion, whereas machine-based mixing provides the extra motion to overcome the unstirred layer effect of passive diffusion reactions on a solid surface such as a tissue section. The speed and uniformity of the reaction is also aided by heating. Although heating ensures rapid acquisition of equilibrium, it comes with a cost: evaporation. Evaporation of reagents results in slide drying, which is the number one enemy of slide-based IHC assays. To obviate this problem, our automated assays are performed utilizing a liquid coverslip or light oil, which covers the reactants and ensures constant buffer volumes. Automated instruments also have the advantage of mixing the reactant (e.g., DAB and peroxidase) in a mechanized fashion. This yields two benefits: (1) contact with a known carcinogen is avoided and (2) human labor and time are significantly reduced. In addition, automated instruments also add the ability to wash in a more controlled and effective manner. This is important, as IHC assays are ultimately judged visually by their signal-to-noise ratio (S/N). Having a mechanized washing procedure not only saves labor but also greatly reduces the background noise, resulting in a better and cleaner assay. Finally, automation entails the use of bar-coded slide labels and code-driven options, which allow the user the choice of different pretreatment steps such as proteolytic enzyme digestion, or the application of different chromogens in the same run.

1.3. Clinical Applications

1.3.1. Lymphomas

Specific patterns of antigenic expression or immunophenotyping profiles characterize individual lymphomas and distinguish them from reactive lym-

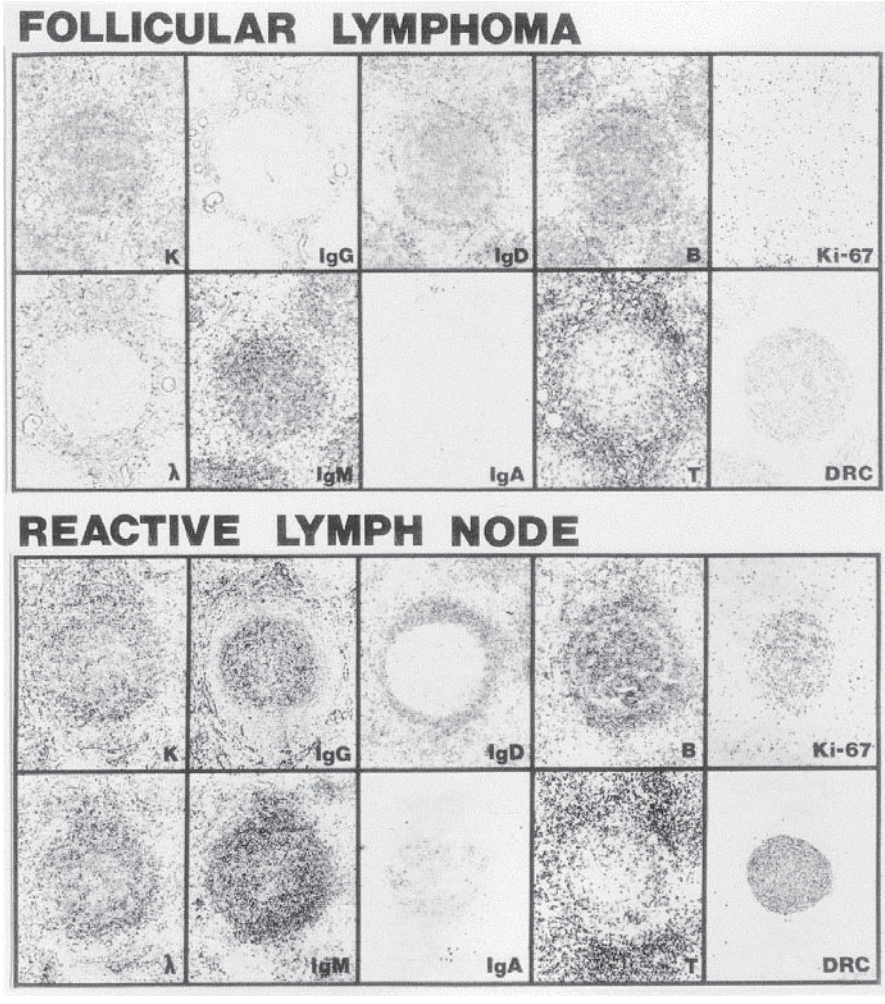

Fig. 3. Reactive lymph node vs. follicular lymphoma. Comparison of monoclonal follicular lymphoma and polyclonal reactive lymph node tissue section phenotypes. DCR, dendritic reticulum cell (detected with anti-CD21); Ki67, proliferation marker (antibody directed at nuclear proliferation protein).

phoid proliferations (*see* **Fig. 3**). Serial sectioning of a tissue blocks allows phenotypic characterization of selected areas within a sample. Large batteries of primary antibodies have been developed for this purpose, and are routinely used in flow cytometry (FCM) and tissue section IHC. The most significant advantage of tissue section IHC is the preservation of the tissue topography, which allows the integration of both morphologic and immunologic properties

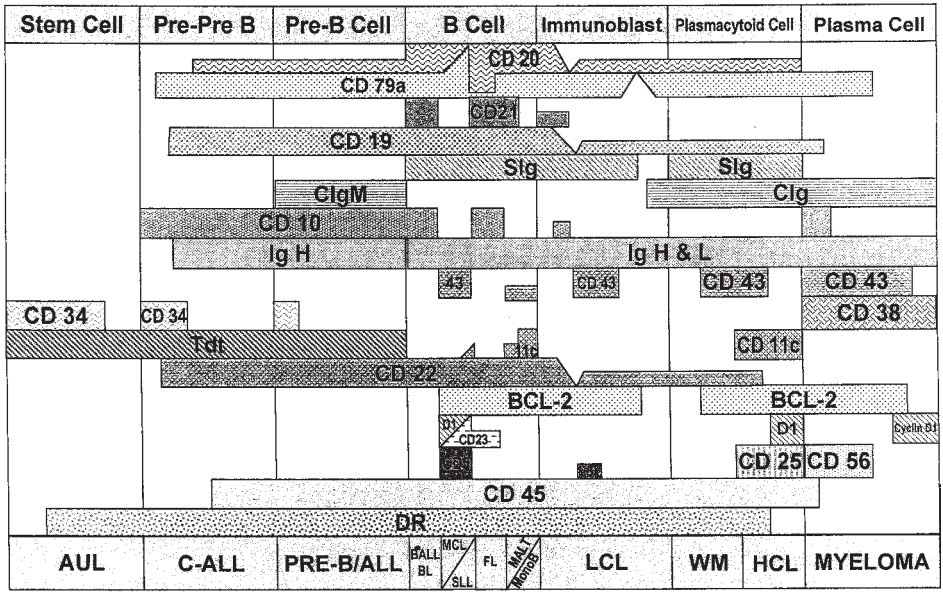

Fig. 4. B-cell phenotypes. Diagrammatic representation of the range of B-cell antigenic and Ig gene expression both in normal B-cell development (ontogeny) and in the B-cell neoplasms derives from each stage of ontogeny. AUL, acute undifferentiated leukemia; C-ALL, common acute lymphoblastic leukemia; PRE-B/ALL, pre B-cell acute lymphoblastic leukemia; B-ALL, B-cell acute lymphoblastic leukemia; BL, Burkitt's/Burkitt's-like lymphoma; MCL, mantle cell lymphoma; SLL, small lymphocytic lymphoma; CLL, chronic lymphocytic leukemia; FL, follicular lymphoma; MALT, mucosa-associated lymphoma; MonoB, monocytoid B-cell lymphoma; LCL, large cell lymphoma; WM, Waldemström's macroglobulinemia; HCL, hairy cell leukemia.

(*see* **Fig. 3**). The end product of this process of integration is the elucidation of distinct immunoarchitectural patterns that characterized most of the lymphomas as biologic entities. This integrative approach has been emphasized by the 1994 Revised European-American Lymphoma (REAL) classification *(2)*. **Figures 4** and **5** depict the different immunophenotypic profiles that can be seen in a wide variety of lymphoproliferative processes of either B- or T-cell lineage. Lymphoma phenotypes generally reflect normal B- or T-cell counterparts with an aberrant twist [e.g., monoclonality, overexpression of oncogenes such as *BCL-2*, *P53*, and cyclin D-1, or pan-T antigen loss (*see* **Fig. 6**)]. Ultimately, all results are interpreted within a matrix of findings, with a typical case requiring between 10 and 15 antibody assays, thus ensuring that diagnosis entails finding a pattern of antigens expressed or absent as illustrated in **Figs. 3** and **4**.

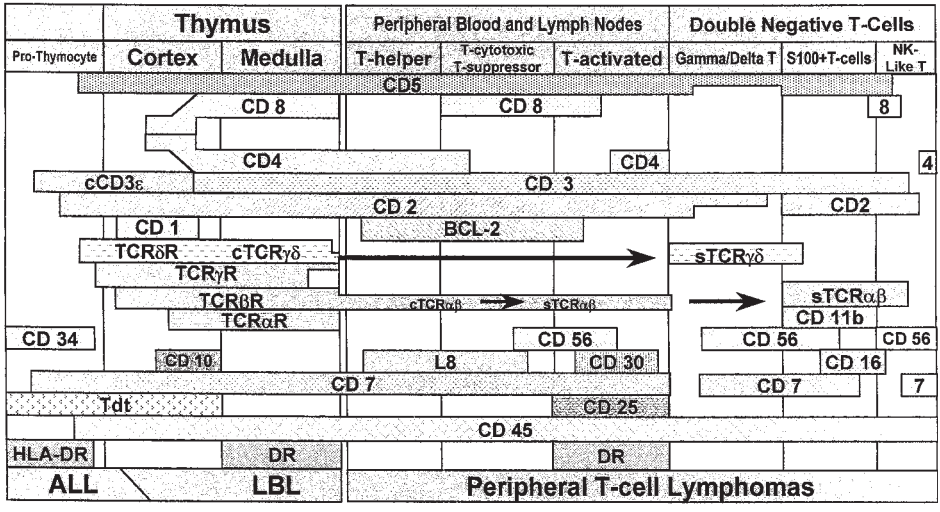

Fig. 5. T-cell phenotypes. Diagrammatic representation of the range of T-cell antigenic and T-antigen receptor (TCR) gene expression both in normal T-cell development (ontogeny) and in the T-cell neoplasms derived from the different developmental stages. ALL, acute lymphoblastic leukemia; LBL, lymphoblastic lymphoma; NK, natural killer; TCR, T-cell receptor.

1.3.2. Leukemias

The preferred method of evaluation of leukemias is flow cytometric analysis of either blood or bone marrow samples. Although ideal for the evaluation of cell suspensions, FCM requires a minimal number of cells ($>10^4$ cells) to be present in order to yield meaningful results. When these requirements are not fulfilled, the paucicellular cell suspensions can be spun down and used in immunocytochemistry cytospin preparations (*see* **Fig. 7**). A case in point, a cerebrospinal fluid sample with rare leukemic cells can be tested for TdT (deoxinucleotydil transferase) via cytospins (*see* **Fig. 7**). Tissue section IHC may also be necessary when bone marrow sampling produces a "dry tap" requiring core or clot section staining for leukemic antigens (*see* **Fig. 7**).

2. Materials

2.1. Specimens

Suitable specimens for immunohistochemistry/immunocytochemistry include:

1. Fresh-frozen or formalin-fixed, paraffin-embedded solid tissue samples.
2. Heparinized samples of blood, bone marrow aspirate, or body fluids.

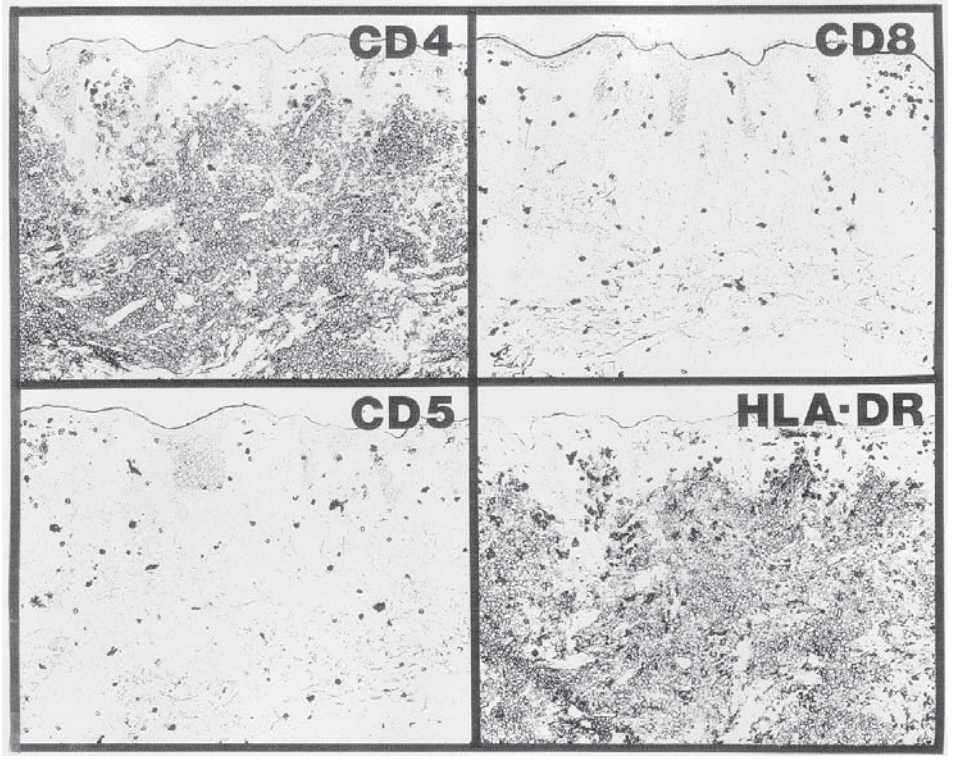

Fig. 6. Peripheral T-cell lymphoma of skin with an aberrant T-cell phenotype: loss of CD5 and CD8, predominance of CD4, and activated status (HLA-DR+).

2.2. Reagents

2.2.1. Specimen Preparation

1. Standard heparin solution.
2. RPMI 1640 (ICN Biomedical).
3. RPMI culture medium at pH 7.2–7.5: 500 mL RPMI 1640 medium without L-glutamine, 10 mL L-glutamine, 10 mL penicillin-streptomycin, 10 mL 1 *M* tricine buffer, 10 mL 7.5% sodium bicarbonate, 5 mL 1 *M* HEPES buffer.
4. LSM (lymphocyte separation medium, ICN Cappel).
5. 20% Fetal calf serum (FCS): 100 mL pure FCS, 400 mL RPMI.
6. Acetone.
7. OCT (ornithine transcarbamoylase compound, Sakura Finetek).
8. 10% neutral-buffered formalin.
9. Xylene.
10. Ethanol alcohol (absolute, 95%, and 80%).

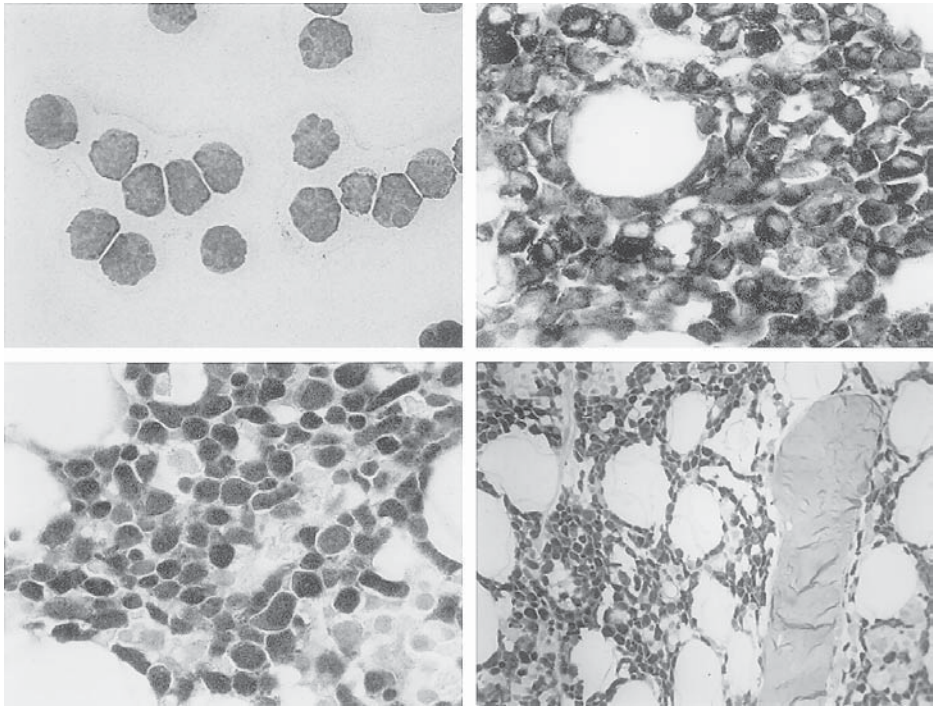

Fig. 7. Leukemia phenotype. Lymphoblasts in cytospin, positive nuclear staining for TdT (upper left); bone marrow core biopsies: myeloblasts staining positive for myeloperoxidase (upper right), lymphoblasts staining for TdT (lower right and lower left).

2.2.2. Manual Immunohistochemistry

1. Monoclonal/polyclonal antibody to human cell surface antigen (primary antibody) (*see* **Table 1**).
2. Antibody diluent (2% bovine serum albumin [BSA] phosphate-buffered saline [PBS]).
3. PBS solution at pH 7.4.
4. BSA working solution: 10 mL of 22% BSA, 90 mL working PBS.
5. 0.02% Stock thimerosal: 20 µg thimerosal, 100 mL distilled water.
6. Acetate buffer at pH 5.0: 6 mL glacial acetic acid, 994 mL distilled water.
7. Biotin-conjugated (biotinylated) goat anti-mouse or goat anti-rabbit secondary antibody diluted 1:200 (93.5 mL PBS, 0.5 mL biotinylated antibody, 1.0 mL stock thimerosal, 5.0 normal human serum).
8. Avidin-D conjugated with HRP (Avidin-HRP) diluted 1:100 (93 mL PBS, 1.0 mL avidin-D, 1.0 mL stock thimerosal, 5.0 mL normal human serum).

9. DAB solution: 3,3-diaminobenzidine tetrachloride): 150 mg DAB, 50 mL PBS, 0.5 mL H_2O_2.
10. Copper sulfate solution: 20 g copper sulfate, 34 g sodium chloride, 4000 mL distilled water.
11. 1% Methyl green solution: 4 g methyl green, 400 mL acetate buffer, HCl to adjust pH to 4.2–4.5.

2.2.3. Automated Immunohistochemistry

1. DAB-HRPO (Ventana Medical Systems, Tucson, AZ).
2. Fast red-alkaline phosphatase.
3. Magnesium chloride solution.
4. Naphthol solution.
5. Fast red A and B solutions.
6. Fast blue-alkaline phosphatase.
7. BCIP solution.
8. NBT solution.
9. HRPO inhibitor solution (H_2O_2 and sodium azide).
10. Alkaline phosphatase inhibitors (L-homoarginine, levamisole).
11. Bar-coded glass slides.

2.2.4. Antigen Retrieval

1. PBS at pH 7.4.
2. Trypsin dilutions 1:250 (0.05 g/100 mL PBS).
3. 1% Glycerin solution (1 mL glycerin, 1 mL PBS).

2.2.5. Avidin Blocking

1. PBS.
2. Avidin (dilution 1:10 with 2% BSA).
3. Biotin (undiluted).

2.2.6. Peroxidase Blocking

1. 3% Hydrogen peroxide solution.
2. PBS solution at pH 7.4.

3. Methods

3.1. Specimen Preparation

The quality of histologic, cytologic, and immunophenotypic material is highly dependent on the promptness and adequacy of tissue handling and fixation. In the case of solid organs, such as lymph nodes and spleen, thorough gross examination and careful sampling are fundamental in selecting the diagnostic material to be studied. Maximum antigen preservation, which is crucial in IHC, requires prompt freezing and proper fixation of the tissue.

3.1.1. Bone Marrow Aspirate, Peripheral Blood, and Body Fluids

Cytospin preparations of cell suspensions can also be successfully used for IHC.

1. Specimen reception: Heparinized samples of bone marrow aspirate, blood, and body fluids are required for cytospin preparations. In general, 0.5 mL of standard heparin solution is added to 6–8 mL of sample (prepackaged sodium heparin green-top tubes are also commercially available). If samples are going to be processed the next day they should be kept refrigerated at 4°C overnight.
2. Specimen dilution: Bone marrow and blood samples are diluted with equal amounts of RPMI tissue medium, before undergoing separation. Other body fluids may or may not require dilution or separation depending on the amount of blood and the density of cells present in the sample.
3. Specimen centrifugation and density separation: Gently layer the diluted sample on top of 4–5 mL of LSM and centrifuge at 1500 rpms for 20–30 min to allow appropriate cell separation. Remove cells from buffy coat and wash them in 5–6 mL of RPMI and centrifuge at 400–450*g* (1500 rpms) for 7–10 min. Discard supernatant and resuspend cells in 4 mL of RPMI to obtain a stock solution. Fill another tube with 6 mL of 20% FCS, and add drops of stock solution until getting a slightly hazy fluid; this represents approx 500,000–1,000,000 cells/mL of suspension.
4. Cytospin preparation: Place slides in the cytospin clips, and add three to four drops of the cell suspension into each cyto cup. Centrifuged at 800 rpm for 2 min, and then allow the slides to air-dry. Subsequently, fix the slides in cold acetone (4°C) for 10 min and let them air-dry; repeat the acetone fixation and air-drying steps before staining. If the slides are not going to be stained in the following 48–72 h, store at –80°C until stained.

3.1.2. Lymph Node, Spleen, Bone Marrow Core Biopsy/Clot Section, and Other Solid Organs

1. Specimen reception: After harvest from the patient, the specimen should be placed in a sterile container and should be kept wet at all times; this is particularly critical when dealing with small tissue samples, which tend to dry out rapidly. A gauze, saturated with 0.9% sodium chloride (saline solution), can be used for this purpose. Thorough gross examination of the specimen by an experienced pathologist or histotechnologist should be followed by prompt preservation and sampling of the specimen.
2. Touch preparations: After thorough gross examination and sectioning of the specimen, a fresh-cut surface is used to make several (5–10) touch preparations. These are dipped in cold acetone, allowed to air-dry, and subsequently stained with a Wright-Giemsa stain.
3. Snap-freezing: Although more and more antibodies can be used on formalin-fixed, paraffin-embedded tissue, there are a small but still significant number of markers that required fresh frozen tissue in order to detect the desired antigen.

Snap freezing entails placing a pea- to almond-sized portion of tissue (not larger than 12 × 12 mm^2 and 4 mm thick) in a cryomold with OCT, and freezing at –150°C for 10 s in either isopenthane quenched in liquid nitrogen, or in liquid nitrogen alone. In addition to ensuring optimal antigen preservation, snap freezing at such low a temperature prevents the formation of ice crystal artifact in lymphoreticular tissues, which can significantly compromise the morphology of the tissue section.

4. Storage: Once frozen, the specimen should be wrapped up in tin foil and placed in an airtight container (plastic zip-lock bag) to avoid tissue dehydration. The tissue should be kept frozen at –80°C in a nondefrosting, centrally alarmed freezer until further studies are performed. Central alarming ensures that tissue will not be lost to power outages or other catastrophes.
5. Frozen sections: At the time of sectioning, the temperature of the tissue should be kept between –20°C and –17°C; bloody specimens need a slightly warmer temperature (–10°C) to keep them from shattering. The tissue should be sectioned as thin as possible, ideally 3 μm thick for optimal morphology. For serial frozen section IHC, the sections are fixed in cold acetone (4°C) for 10 min, then allow to air-dry, and stored in a dessicator at room temperature until stained. If no immunostaining is going to be performed in the following 48–72 h, the sections can be wrapped in aluminum foil and stored at –80°C. To defrost slides, place them in the 60°C oven for 3 min or at room temperature for 10 min.
6. Fixation: Dime-thickness (2 mm) tissue sections are ideally fixed in 10% neutral buffered formalin for 4–8 h, processed overnight in an automatic tissue processor, and then embedded in paraffin. Small tissue blocks are preferred, as they undergo a more uniform fixation, which ensures a more even immunostaining. Paraffin blocks can be stored for long periods of time at room temperature without significant loss of the antigenic properties of the embedded tissue; this has allowed the study of archival material several decades old.
7. Permanent sections: Multiple thin-tissue sections (3μ thick) are required for both hemaloxylin and eosin (H&E) and IHC stains. A thin section allows evaluation of fine nuclear and cellular detail, ensuring a precise morphologic characterization of the process under study, which is fundamental in the selection of the battery of markers to be used.
8. Deparaffinization:
   a. Deparaffinize slides in xylene (10 min in each one of two xylene dishes).
   b. Place in absolute ethanol, 5 min in 95% ethanol, and 5 min more in 80% ethanol.
   c. Rinse in tap water for 5 min.

3.2. Manual Immunohistochemistry (3,1)

1. Prepare slides as described in **Subheading 3.1.**
2. Apply the selected antibodies to the corresponding slides (80–100 μL depending on the size of the specimen). A new pipet tip is used for each antibody.

3. Incubate in sealed moist chamber for 20–25 min.
4. Wash slides with PBS and drain off excess.
5. Immerse slides in biotinylated secondary antibody dilution for 20 min; increase time if necessary.
6. Wash slides thoroughly (both front and back) with PBS.
7. Immerse slides in Avidin-HRP for 20 min; increase time if necessary.
8. Wash slides with PBS, and then immerse them in PBS.
9. Incubate slides in DAB-HRPO for 5 min in the dark.
10. Place slides in a slide rack, rinse in PBS, and dip them 10 times in two changes of distilled water.
11. Incubate in 0.5% copper sulfate in 0.85% sodium chloride for 5 min.
12. Rinse in two changes of distilled water, air dry and coverslip. If counterstain is desired, modify **step 12** as follows:
13. Rinse in two changes of distilled water, and then immerse slide in acetate buffer (pH 5) for 5 min, and then in methyl green solution for 2–7 min.
14. Rinse in 90% acetone (10 dips), and then in 100% acetone (10 dips).
15. Place slides in two changes of xylene and then coverslip.

3.3. Automated Technique

1. Prepare slides as described in **Subheading 3.1**.
2. Slides are then bar coded (*see* **Subheading 1.1.4.**).
3. After full automated staining, the slides are manually removed from the instrument and coverslipped.

3.4. Antigen Retrieval

The major deficiency of paraffin IHC assays is the masking of epitopes by crosslinking fixatives, which can completely block the antigen–primary antibody binding. Several strategies have been developed to overcome this problem. One entails selecting monoclonal antibody probes to epitopes that are fixation resistant. Additional techniques involve unveiling the crosslinked epitope by either proteolytic enzyme digestion with trypsin/proteases, and/or acid hydrolysis with microwaving ***(4)***. These techniques may enhance the detection rate of certain antigens in paraffin sections. Specific combinations of enzyme digestion and microwaving may be used depending on the epitope to be unmasked and the antibody to be applied. It is important to remember that some antigens are not affected by fixation, and therefore do not require unmasking; moreover, in certain cases antigen retrieval may even be even contraindicated. In addition, excessive exposure to enzyme digestion or microwaving can be deleterious to the tissue, compromising both the morphology and the quality of the staining ***(5–7)***. The tissue slides should undergo unmasking before the actual immunostaining procedure begins. For trypsin digestion, the procedure is as follows:

1. Prepare slides as in **Subheading 3.1.**
2. Place slides in 1:250 trypsin solution for 10 min at room temperature.
3. Rinse in PBS.
4. Place slides in 1% glycerin for 1 min at room temperature to stop the digestion reaction.
5. Rinse in PBS.
6. If microwaving is also required, at this point proceed as described by the manufacturer's protocol (*see* Antigen Retrieval and Standardization, Biogenex, San Ramon, CA).
7. Proceed with the immunostaining run according to procedure (*see* **Subheadings 3.2.** and **3.3.**).

3.5. Avidin Blocking Step

Certain tissues are capable of binding avidin, biotinylated HRP, or other biotin/avidin compounds without previous addition of the biotinylated antibody. This phenomenon is likely due to the presence of endogenous biotin or biotin-binding proteins present in the tissue section. In order to avoid this nonspecific reaction, the slides can be pretreated as follows:

1. Prepare slides as described in **Subheading 3.1.**
2. Incubate slides with avidin for 15 min in a moist chamber.
3. Rinse with PBS, and then wipe excess off slides.
4. Incubate with biotin for 15 min in a moist chamber.
5. Rinse with PBS, and then wipe excess off slides.
6. Add primary antibody and continue according to protocol (*see* **Subheading 3.2.**).

3.6. Endogenous Peroxidase Blocking

If HRP is going to be used as the enzymatic component in the labeling system, nonspecific staining due to the presence of naturally occurring endogenous peroxidase can become a significant problem. Blockade of the endogenous peroxidase is routinely applied to reduce the resulting noise. The procedure is as follows:

1. Prepare slides as described in **Subheading 3.1.**
2. Prior to immunostaining, pretreat the deparaffinized slides with 3% H_2O_2 for 10 min at room temperature (use 3% H_2O_2 in methanol for bloody specimens).
3. Rinse the slides with PBS.
4. Add primary antibody and continue according to protocol (*see* **Subheading 3.2.**).

3.7. Controls

It is fundamental to ensure that the primary antibody is yielding specific staining of the target antigen and that the pattern of staining is the expected one. Every time an IHC staining run is performed, both positive and negative

controls are run simultaneously in order to document the sensitivity and specificity of the staining process. The control tissue can be placed in the same slide with the section to be studied; care should be taken to select a control tissue that was fixed with the same fixative used in the study sample.

3.7.1. Positive Control

The positive control allows us to evaluate the specificity of the primary antibody and the intensity of the IHC signal on a given assay. To accomplish this, we typically employ a section of tonsil, which is positive for most of the antibodies listed in **Table 1**. This control is at the base of the slide and represents the "same" slide positive control. We occasionally use a control slide with a portion of thymus, which serves as positive control for TdT and CD1a, or a cytospin of a cell line for leukemic antigens (NALM6) or oncogenes (*MCF7, P53*).

3.7.2. Negative Control

The negative control is performed to ensure that in the absence of the primary antibody no IHC signal is present. Any immunostaining resulting from this procedure represents nonspecific staining (noise). If the level of noise is significant, modifications to the protocol are needed. Depending on the assay, our negative control consists of the following: (1) the primary antibody is replaced by an equal volume of buffer, or (2) the primary antibody is substituted by an irrelevant isotype-matched antibody.

3.7.3. Tissue Control

The immunogenicity of the tissue under study should also be tested; the most common strategy is the use of vimentin. Vimentin is an intermediate filament present in a wide variety of mesenchymal cell lineages; therefore, virtually any tissue sample will have some expression of this marker in association with blood vessels, nerves, and connective tissue in general. If vimentin is completely absent, the antigenicity of the sample should be seriously questioned.

4. Notes

1. False positive staining: Because all antibodies, even monoclonal ones, crossreact with other molecules than the specific target molecule, care should always be taken to exclude false positive staining. In general, "false positive" staining occurs as a weak signal, thus strong reactivity is reassuring for likely specificity. But the "same" slide control is also very important, as it typically provides a microanatomic measure of specificity. For example, in judging the true positivity of the signal for cyclin D1 in tissue section IHC, we utilize the "same" slide section of tonsil, wherein the basal cell nuclei of the tonsillar epithelium express physiologic levels of cyclin D1; any weaker staining is taken to be nonspecific.

2. False negative staining: This may occur under several circumstances: (1) failure to apply reagents per protocol, (2) application of out-of-date, contaminated, or spent reagents, or (3) improper fixation with masking of the target. For the diagnostician, the lack of reaction of the same slide tissue control should be diagnostic of these circumstances, as no reaction will have occurred. The most difficult circumstance alone is improper fixation, which may not be matched by the same slide control. Because of this uncertainty, a totally negative reaction must be received with great skepticism, and typically other means of tissue unmasking should be pursued (e.g., enzymes, microwaving, change of pH).
3. Fixatives other than formalin: Although formalin is the most common fixative for immunohistochemical procedures, other fixatives can be successfully used as well. Some of the frequently encountered fixatives include: B-5, Bouin's solution, alcohol-based fixatives (e.g., Carnoy's, Omni-Fix), zinc formalin-based fixatives (e.g., Z-Fix), and PLP (periodate-lysine-paraformaldehyde). The procedure is essentially the same, however, minor adjustments in the immunostaining protocol may be required (e.g., incubation times, antibody titers, antigen unmasking techniques). In addition, both positive and negative controls should be fixed with the same fixative used in the tissue under evaluation.
4. Incubation time: In general, the longer the incubation time, the more intense the signal. Unfortunately, the nonspecific background staining or noise also increases. For these reasons, the incubation with the primary antibody time varies depending on the antigen being used, and has to be modified in order to obtain the optimal signal-to-noise ratio. In the case of commercially available antibodies, following the manufacturer's recommendations is usually a good starting point.

   When using a manual technique, prolonged incubations can be associated with an increased chance of sections drying out. To prevent this phenomenon, the incubation chamber has to be kept moist and properly sealed during the incubation time.
5. Decalcification: Decalcification is necessary when dealing with bone samples (e.g., bone marrow core biopsies) or with markedly calcified soft tissues. IHC can be successfully performed in decalcified specimens provided that complete fixation of the biopsy has occurred prior to decalcification (*see* **Fig. 7**). Occasional antigens (e.g., Ki67) are not tolerant of decalcification, and alternative clones have to be used (e.g., MiB1).
6. Poor morphology: Poor morphology is likely to result from three common circumstances: (1) poor fixation, (2) tissue cutting with a dull blade, and (3) overdigestion with enzymes. Obviously, the attempt to correct for poor fixation and cutting with a dull blade by using an excess of enzymes only compounds the problem. The answer lies in strict adherence to protocol for sections (*see* **Subheading 3.1.**).
7. Ice crystal artifact: Freezing artifact occurs when lymphoreticular tissue is not properly frozen. It manifests as multiple polygonal white spaces in the tissue section, and occurs when the specimen is frozen slowly allowing for entrapment of water in the extracellular space. To avoid this artifact, lymphoreticular samples

should be snap frozen at very low temperatures (–150°C). If ice crystal artifact has already occurred, the tissue can be thawed and refrozen at –150°C as previously described (*see* **step 3** in **Subheading 3.1.2.**).

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5

Molecular Genetics and Cytokines

15

Apoptosis

Molecular Regulation of Cell Death and Hematologic Malignancies

Vincenzo Chiarugi, Marina Cinelli, Lucia Magnelli, and Persio Dello Sbarba

1. Introduction

1.1. Molecular Mechanisms of Genetically-Programmed Cell Death

Apoptosis, or programmed cell death, represents in cell biology a functional program as important as cell growth or differentiation. Programmed cell death is of basic importance for the development of multicellular organisms and its basic mechanisms are conserved during the evolution of metazoa. Mammalian cells exhibit several different apoptotic pathways that converge to a common endpoint. Each pathway is triggered by a different stimulus: growth factor default, irradiation, induction of the p53 oncosuppressor protein, glucocorticoid hormones (in lymphocytes), ligand binding to Fas/APO (CD95), or tumor necrosis factor receptor (TNF-R), perforin secreted by cytotoxic T cells (reviewed by Hale et al. ***[1]***). As opposed to necrosis, apoptosis is a "clean" process: as the cell shrinks, the cell membrane turns into the "apoptotic shell," the nucleus is condensed and reduced in volume, and eventually the cell disappears from the tissue, due to phagocytosis by neighboring cells or professional phagocytes, such as macrophages.

Apoptosis proceeds along subsequent phases. The earliest phase is represented by the generation of the stimulus that triggers the apoptotic response. The second phase is the transduction of this stimulus by specific cellular sensors and the transmission of this signal to the cell death effector machinery.

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The third phase includes the activation of this machinery, composed of a series of proteases, as well as of their positive and negative regulators. A fourth post-mortem phase includes chromatin condensation and degradation, membrane coartation by transglutaminases, and phagocytosis ***(2)***.

The mechanisms of cell death in mammals is based on a complex cascade of cysteine proteases, which are preformed and stored in the cytoplasm as precursors molecules, and are ready to be activated without need to be newly synthetized ***(3)***. This basic cell-killing machinery was discovered in vertebrates due to the homology with an apoptotic gene of *Caenorhabditis elegans*, a nematode worm, and named CED (CEll death abnormal)-3 ***(4)***. In mammals, six different homologs of *CED*-3 have been identified and named ICE (interleukin-1β–converting enzyme; or TX, or ICH-2), ICErel-II, ICErel-III (or TY), CPP-32, *Mch*-2, and NEDD-2 ***(5–7)***. All these genes encode for cysteine proteases, which are translated as inactive precursor polypeptides. These precursors are activated by cleavage at aspartate residues as a consequence of the triggering of apoptotic stimuli and then assembled into heterodimers or heterotetramers. All these cysteine proteases cleave their substrates at an aspartate residue, but within a specific aminoacid sequence, which determines the substrate specificity ***(8)***. Mature ICE can activate pro-ICE, as well as pro-CPP32 ***(3)***. The cellular substrates of these cell-killer proteases are poorly known. It may be that there is a large number of them or, to the contrary, a limited number of substrates that must be cleaved for cell death to occur. CPP32, for instance, can cleave and inactivate poly-ADP-ribose polymerase (PARP), an enzyme necessary for DNA repair ***(9)***. PARP cleavage is considered as a marker of apoptosis, but the significance of the hydrolysis of a DNA repair enzyme is not yet understood. It is possible that since DNA must be extensively degraded during apoptosis, DNA repair enzymes must consequently be eliminated.

The regulation of the cell-destroying machinery based on cysteine proteases is generally believed to be carried out by ancestral proteins of the *Bcl*-2 family. As in the case of CED-3 and ICE, there is a strict homology between the antiapoptotic gene of *C. elegans* CED-9 and mammalian *Bcl*-2. *CED*-9 can be substituted by the human *Bcl*-2, thus demonstrating that the gene does not change significantly during the evolution from nematodes to humans ***(3)***. The mechanism of action of the products of *Bcl*-2 and *Bcl*-2-related genes has been extensively pursued in recent years with a series of contradictory results. What we know is that *Bcl*-2 forms a dimer with itself or with other members of the family. The homodimer *Bcl*-2/*Bcl*-2 inhibits apoptosis, whereas *Bax*/*Bax* activates it; the heterodimers *Bcl-xs/Bcl*-2 and *Bcl-xL*/*Bcl*-2 result in the block or the stimulation, respectively, of *Bcl*-2 action ***(1)***. What is still unknown are the molecular targets of the regulatory activity of *Bcl*-2 family gene products on

the apoptotic process. It has been proposed by Vaux and Strasser ***(2)*** that the *Bcl*-2 family gene products regulate the central apoptotic machinery, i.e., the cysteine–protease cascade. This is very probable, due to the wide (although not universal) inhibitory activity of *Bcl*-2 on a variety of apoptotic pathways.

A special place in the complex regulation of the apoptotic processes is reserved to Fas/APO-1 (also known as CD95) and TNF-R. The receptors of the TNF-R family are characterized by the presence of cysteine repeats in their extracellular domain and exhibit similarity with the cell death protein "reaper" of *Drosophila* ***(10)***. These receptors form, with their intracellular domains, alternative clusters with different cytoplasmic factors. These phenomena result in cell life or death, depending on the alternative activation of caspase-8, a member of the *ICE* family, or NFκB, a nuclear transcription factor very important in the immunohematopoietic system ***(11)***.

The proteins that form complexes with the intracellular domains of CD95 and TNF-R have begun to be identified. The receptors form dimers after interacting with ligands. Activation of CD95 triggers the intracellular recruitment of Fas-associated protein with death domain (FADD) and caspase-8, which acts as a linkage between the signaling complex and the ICE–protease cascade. Fas-associated phosphatase 1 (FAP-1) can also be included in the complex as a negative regulatory element that blocks apoptosis ***(12)***. The recruitment of elements by the intracellular domain of TNF-R is even more complex: the TNF-R-associated death domain (TRADD) protein probably acts as a docking protein that may mediate the two diverse pathways of life and death. In the "death" pathway, the TRADD protein interacts with FADD/MORT-1 (mediator of receptor-induced toxicity), thereby activating caspase-8. In the "life" pathway, TRADD may recruit receptor-interacting protein (RIP), a serine–threonine kinase containing a death domain), which, via its kinase domain, binds TNF-R-associated protein/factor (TRAP/TRAF-2), which in turn activates NFκB and supports cell survival. The picture is far from being clear, but the discovery of receptors involved in both cell life and death is very important, although their regulation in one direction or another is still to be discovered ***(12)***.

The roles of Fas/APO and TNF-R is particularly relevant in hematopoietic diseases. Nagata ***(13)*** presents the more up-to-date model for Fas ligand- and TNF-mediated apoptosis. Binding trimeric cytokines to their receptors induces receptor trimerization. FADD/MORT-1 binds to the trimerized death domain of the Fas cytoplasmic region. Caspase-8 is then recruited through the binding to the death domain of FADD/MORT-1; caspase-8, in turn, activates itself and may activate other caspases. The recruitment of caspase-8 by TNF-R1 occurs via the binding of TRADD to the death domain of TNF-R1. TRADD, in turn,

may recruit FADD-MORT-1 or RIP, via its death domain, resulting in apoptosis. However, as we explained earlier, TRADD may also recruit RIP in association with TRAP/TRAF-2, with the consequent NFκB activation and cell survival.

Death factors like Fas-ligand and TNF are useful in maintaining homeostasis of the immunohematopoietic system. However, if the system is under- or overexpressed, it has deleterious effects. For instance, loss of function causes hyperplasia, such as lymphoproliferation. Although the heterozygous mutation in the *Fas* gene is not tumorigenic, it may lead to Hodgkin's lymphoma ***(14)***.

On the other hand, when the system overfunctions, it causes tissue distruction. When *Fas* is overexpressed in mice and massively activated in vivo, the mice die of fulminant hepatitis ***(15)*** caused by abnormally activated T cells. Also, the transformation of hepatocytes with hepatitis B or C viruses causes the upregulation of the *Fas* system expression ***(13)***.

An important function of the Fas/TNF-R systems is to kill cells bearing viral antigens at their cell surface. When this system is excessively active, it may lead to fulminant tissue death. Under this light, it is also possible that other CTL-induced diseases, such as graft-versus-host disease, AIDS, and immune insulitis are also mediated by the Fas/TNF-R system.

In summary, the apoptotic process can be tentatively divided in the following phases:

1. Apoptotic stimuli: GF default, irradiation, c-*myc* overexpression, glucocorticoid treatment (in lymphocytes), activation of Fas/APO and TNF-R.
2. Detectors of apoptotic stimuli: receptors for GF and TNF, Fas/APO, p53, glucorticoid receptors in lymphocytes, perforin (CTL).
3. Effectors (cascade of cysteine-proteases): ICE (caspase-1), ICErel-II, NEDD-2, CPP-32 (caspase-3), granzyme, other caspases.
4. Negative effector regulators: *Bcl*-2, *Bcl*-xL, *Bcl*-w, *Mcl*-1.
5. Positive effector regulators: *Bax*, *Bcl-xs*, *BAD*, *Bak*, *Bik*.
6. Cell-destroying enzymes: PARP (inactivated), hydrolase, nucleases, transglutaminases.

2. The Involvement of Apoptosis in the Genesis of Immunohematopoietic Diseases

Apoptosis is an important aspect of the physiology of the immunohematopoietic system. For instance, apoptosis is involved in the suppression of self-reactive lymphocytes ***(16,16a)*** and in the modulation of reactivity of hematopoietic cells to growth factors and cytokines ***(17)***. CTL are also well known to induce apoptosis in their target cells with the perforin/granzyme-B system ***(17a)***. Also some cytokines, such as TGF-β (transforming growth factor) and TNF-α, are inducers of apoptosis. The regulation of apoptosis is very peculiar

and seems largely dependent on the target cells: it protects monocytes and granulocytes from apoptosis, while inducing apoptosis in leukemic cells.

The dysregulation of apoptotic processes in the immunohematopoietic system causes the emergence of a variety of diseases, due to either apoptosis inhibition or "excessive" apoptosis. Apoptosis inhibition characterizes follicular B-cell lymphomas, chronic lymphocytic leukemia (CLL), blast crisis of chronic myelogenous leukemia (CML), infectious mononucleosis, Burkitt's lymphoma, and systemic lupus erythematosus (SLE). An excessive apoptosis of specific cell clones, on the other hand, is involved in myelodysplastic syndromes, β-thalassemia and acquired immunodeficiency syndrome (AIDS).

The classic example of hematopoietic malignancy etiologically related to the inhibition of apoptosis is the follicular B-cell lymphoma, associated with a 14 : 18 translocation that involves the *bcl*-2 gene. This gene, normally located in the long arm of human chromosome 18, is translocated to chromosome 14, close to the immunoglobulin heavy-chain enhancer ***(18)***. This determines a severalfold increase of the expression of the Bcl-2 protein and the consequent shift of the equilibrium between Bcl-2 monomers and dimers in favor of the dimers. This results in the inhibition of the apoptotic process and in the accumulation of B cells in the lymphatic follicle, followed by additional oncogenetic hits and the development of a fully malignant status.

Infectious mononucleosis and Burkitt's lymphoma represent other important cases in which an impaired apoptosis characterizes the onset of disease. The etiological agent of mononucleosis, the Epstein-Barr virus (EBV), induces the immortalization of B lymphocytes, which are cells physiologically programmed for a limited life span. The viral gene *BHRF*-1 has sequence homology with *bcl*-2 and is, therefore, suspected to determine inhibition of apoptosis and a consequent excessive lymphocyte proliferation ***(19)***.

Burkitt's lymphoma is typically characterized by an 8 : 14 translocation, which leads to c-*myc*. overexpression. However, the onset of this lymphoma involves other mechanisms, among which are probably apoptosis inhibition and cell immortalization. These phenomena may be determined, for instance, by the expression of the above-mentioned, *bcl*-2-like gene, *BHFR*-1. It is more likely, on the other hand, that other EBV-derived genes are relevant for the genesis of Burkitt's lymphoma. Peculiar genes are expressed in the course of the EBV "latent" infection of B cells, such as LMP-1, which is associated with the increase of *bcl*-2 expression and acquisition of resistance to the triggering of apoptosis by wild-type p53 ***(20)***. In addition to LMP-1, other latent EBV proteins, called EBNA-2 and EBNA-4, up-regulate the expression of *bcl*-2 ***(21)***. The fact that EBV-carrying Burkitt's lymphomas do not express LMP-1 suggests that the expression of latent EBV proteins may be relevant in the very early phases of oncogenesis, i.e., the immortalization of B cells.

In other immunohematopoietic diseases, inhibition of apoptosis occurs as a second event, determining the establishment of a full malignant status. In the early stages of a subset of CLL, for instance, the lifespan of B-cell clones is abnormally prolonged via the persistent retention of *bcl*-2 expression, which is responsible for the inhibition of apoptosis and is sustained by paracrine IL-4 of T-cell origin. This phenomenon follows an earlier phase in which an increased transcription of the *bcl*-1 gene, due to an 11 : 14 translocation, determines in B cells the autocrine production of growth factors, including TNF-α, IL-1, and IL-6.

The picture is different in the "blast crisis" of CML. CML cells, although capable of surviving and proliferating independently of environmental control, due to the expression of p210*bcr-abl* tyrosine kinase, are normally subjected to apoptosis and terminal differentiation, depending on the activity of wild-type p53 ***(22)***. When in these cells p53 undergoes a mutation with loss of function, the apoptotic process is blocked and the cells proceed to the more indifferentiated state characteristic of blast crisis ***(23)***.

Finally, patients who have SLE exhibit elevated serum levels of a secreted form of Fas ***(24)***. Soluble Fas may competitively inhibit Fas/Fas–ligand interaction, resulting in a reduced Fas-mediated apoptosis and in the consequent accumulation of autoimmune cells. Although no human autoimmune disease has so far been shown to directly depend on the dysregulation of apoptosis processes, transgenic mice overexpressing *bcl*-2 in their B cells develop an SLE-like autoimmune disease ***(25)***.

Other important diseases are linked to excessive apoptosis during the maturation of immunohematopoetic cells, including myelodisplastic syndromes, characterized by an impairment of bone marrow cell maturation resulting in ineffective hematopoiesis associated with morphological changes, and peripheral cytopenias ***(26)***. Two subtypes of this syndrome, refractory anemia (RA) and RA with ringed sideroblasts (RARS), result from an increased apoptosis of CD34+ bone marrow cells (i.e., hematopoietic progenitor cells). This increase is determined by the inhibition of *Bcl*-2 function or *Bax* upregulation ***(27)***. Interestingly, the degree of apoptosis correlated with the *Myc : Bcl*-2 ratio, in keeping with the fact that *Bcl*-2 is an inhibitor of *Myc*-induced apoptosis ***(26)***.

Ineffective erytropoiesis is also an aspect of β-thalassemia (Cooley's anemia), in which erythroblasts show a DNA degradation pattern typical of apoptosis. This suggests that an enhanced apoptosis of erythroid progenitor and precursor cells is a major factor in the pathogenesis of this disease ***(28)***.

AIDS is a very complex disease caused by a retrovirus, HIV, which determines the expression of a number of genes the functions of which are still unknown. The development of AIDS has been directly correlated with the depletion of $CD4^+$ helper T lymphocytes. This depletion appears related to the binding of cell-surface CD4 molecules by the soluble viral protein gp120,

resulting in an enhanced probability of T lymphocytes to undergo apoptosis. This phenomenon physiologically occurs when CD4 receptors are crosslinked before the engagement of T cell receptor by the antigen *(29)*. AIDS T cells, in fact, die due to an active process that is prevented by inhibitors of protein synthesis and has all the features of apoptosis *(30)*.

3. Methods

All the methodologies listed can be applied using commercially available kits from several companies. As all technical details are provided there, we just describe here the basic principles of each method.

3.1. Morphology

Apoptosis was originally defined according to the morphological aspect that distinguishes it from necrosis: cell volume reduction (shrinkage), nuclear condensation, chromatin clumping, and presence of apoptotic bodies. The morphological methods are not different from routine histology. Apoptotic cell DNA shows a markedly increased sensitivity to denaturation, thus staining with metachromatic dyes like acridin orange allows an easy detection of apoptotic cells. On the contrary, necrosis is characterized by cell and organelle swelling and plasma membrane damage, features easily distinguishable from those of apoptosis. A characteristic picture of hematopoietic cells stained by May-Grünwald-Giemsa during the apoptotic process is shown in **Fig. 1** *(31)*.

3.2. DNA Ladder

A postmortem event occurring in apoptosis is the activation of specific endonucleases that cut DNA between nucleosomes. The nucleosome contains 160/200 base pairs wound around a protein core of histones. The activation of endonucleases results in DNA degradation into nucleosomes, so that agarose gel electrophoresis of DNA obtained from apoptotic cells shows a “ladder” of fragments corresponding to multiples of the 160/200 base-pair nucleosomal unit. Monomers (one unit) migrate in the gel as the first band, which is followed by those of dimers, trimers, tetramers, and so on. DNA is routinely extracted by the classic phenol method, cleaned by centrifugation and than subjected to electrophoresis. DNA bands are revealed under UV light after propidium iodide staining. A characteristic picture of DNA ladder from hematopoietic cells is shown in **Fig. 2** *(31a)*.

3.3. Flow Cytometry

Flow cytometry (FCM) on the basis of cell DNA content provides quantitative data on the percentage of apoptotic cells in a cell population. Because of their content of degraded DNA, apoptotic cells appear as a hypodiploid popu-

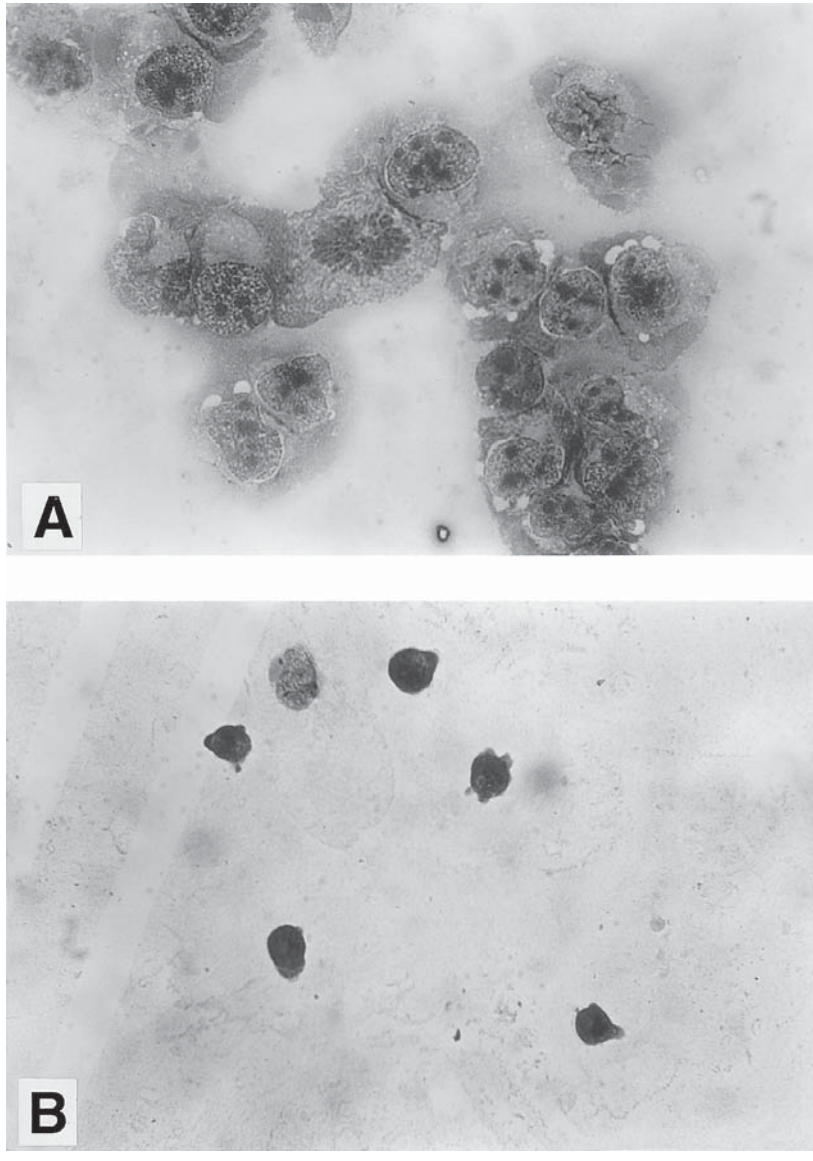

Fig. 1. Morphological features of apoptosis in interleukin-3-dependent 32D leukemia cells, following cytokine withdrawal. After 3 d the apoptotic process is evident in cells lacking the growth factor, as revealed by light microscopy examination. **(A)** Control. **(B)** Apoptosis. From **ref. *31***.

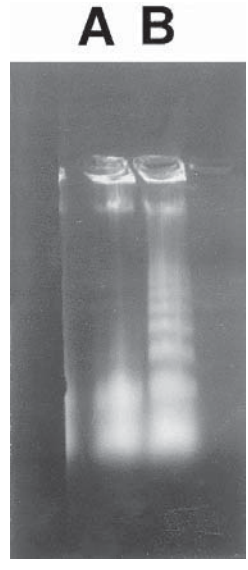

Fig. 2. DNA ladders in agarose of necrotic and apoptotic K562 leukemia cells. DNA was extracted and processed as indicated in **Subheading 3.** and revealed under UV light after staining with ethydium bromide. **(Lane A)** DNA smear of necrotic cells. **(Lane B)** DNA ladder of apoptotic cells. From **ref.** ***31a***.

lation that is eluted before the normal 2N cells in the G1 phase of cell cycle, and has been therefore named the "sub-G1" (or "A0") region of the elution pattern. **Figure 3** shows an unpublished result of our laboratory where the elution position and the percentage of apoptotic cells is evident.

3.4. TUNEL

The method of TdT-mediated dUDP-biotin nick-end labeling (TUNEL) is based on the specific binding of deoxynucleotidyl transferase (TdT) to the 3′-OH ends of DNA strand breaks. The enzyme catalyzes a template-independent addition of deoxyribonucleoside triphospates to DNA. The exposure of nuclear DNA in histological sections to TdT after proteolytic treatment is used to incorporate biotinylated deoxyuridine at sites of DNA breaks. The signal is then amplified by avidin-peroxidase, enabling conventional histochemical identification by light microscopy ***(32)***.

3.5. Direct Detection of DNA Strand Breaks

Exposure to TdT is used to catalyze the addition of fluorescent deoxyuridine triphosphate nucleotides (e.g., F-dUTP) to the 3′-OH ends of double- or single-

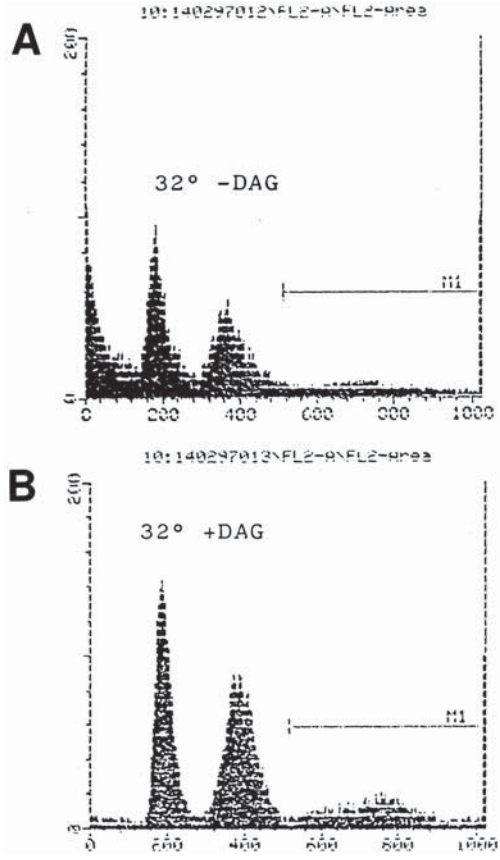

Fig. 3. Flow cytometric analysis of human fibroblasts transfected with a vector containing TS mutant p53 (val 135) expressing wild-type p53 at 32°C and mutated inactive form at 37°C. The study concerns the protective effect of diacylglycerol (DAG) on p53-dependent apoptosis. From left to right, the first peak near the origin represent apoptotic cells, followed by G1 and G2 peaks. The chronic presence of DAG, downregulating protein kinase C, and consequently p53 protects cells from apoptosis (disappearance of peak 1) and induces genome derangement at the chromosomal level in the form of aneuploidity (fourth peak).

stranded DNA. A single-step flow cytometric method has been developed by labeling the the 3′-hydroxyl termini of apoptotic DNA strand ends with BUDR-dUTP, which is more readily incorporated into the genome of apoptotic cells than are larger ligands like fluorescein and biotin. The combination of this method with the use of fluorescein-labeled anti-BUDR monoclonal antibodies generates a stronger signal ***(33)***.

3.6. Single-Stranded DNA Monoclonal Antibodies

Staining cell suspension and tissue sections with monoclonal antibodies (mAb) raised against single-stranded DNA (ssDNA) is a specific and sensitive method to detect apoptotic hematopoietic cells, ideal for the early phases of apoptosis. These antibodies do not recognize double-stranded DNA, and the signal they generate is independent of internucleosomal DNA fragmentation. In contrast with TUNEL, mAb to ssDNA recognize specifically apoptosis at different stages and do not detect necrotic cells. Detection of apoptosis by mAb includes two main steps: (1) fixed cells are heated to induce DNA denaturation *in situ*; (2) single-stranded DNA regions recognized by mAb specifically label apoptotic nuclei, due to the decreased stability of apoptotic DNA to termal denaturation ***(34)***.

3.7. Annexin-V

Recently, a new method for detection of apoptosis in individual cells by FCM has been described. The anticoagulant annexin-V is a member of a family of structurally related proteins that exibit Ca^{2+}-dependent phospholipid binding proteins. Annexin-V binds to various phopholipid species with the highest affinity for phosphatidyl-serine (PS), which in normal hematopoietic cells is located in the inner leaflet of plasma membrane. PS is translocated to the outer layer of the membrane (i.e., to the cell suface) in the early phases of apoptosis, during which cell membrane itself remains intact. At this point, PS can be revealed by ligation with fluorescein isothiocyanate (FITC)-labeled annexin-V and used to quantify apoptotic cells. As necrosis, in contrast to apoptosis, is accompanied by loss of cell membrane integrity and leakage of cell material into the environment, the use of the propidium iodide exclusion test in combination with annexin-V binding represents an ideal procedure to discriminate between apoptosis and necrosis. This method has been applied to several cell types ***(35)***.

3.8. Caspases Inhibitors and Substrates

A number of caspase inhibitors, as well as specific substrates, have been developed. More than 11 caspase inhibitors are now commercially available only for caspase-1 (ICE) ***(36)***. Other important caspases are caspase-2 (Nedd-2), caspase-5 (ICE-3) and caspase-8 (Mach, FLICE) (for review, *see* **refs. *37*** and ***38***).

3.9. Granzyme-B

Granzyme-B is a 27kDa serine-protease stored in granules of activated cytotoxic T cells and natural killer (NK) cells. Upon target cell contact, granzyme-B is directionally exocytosed and penetrates the target cell, where

activates various pro-caspases, thereby inducing apoptosis. Both granzyme-B itself and chromogenic substrates are commmercially available ***(39)***.

3.10. TNF-α Receptor

TNF receptors (TNF-R) are implicated in many immuno-hematopoietic processes. Two distinct TNF-Rs that interact with the ligand TNF-α activate NFκB and Jak kinases, as well as cell death signals. Enzyme-linked immunosorbent assay (ELISA) kits and mAb are commercially available ***(40)***.

3.11. The Fas (CD95) Ligand

Due to the uncertain specificity of available Fas–ligand polyclonal and monoclonal antibodies, the simultaneous use of several Fas–ligand antibodies is recommended ***(41)***.

A recent pubblication by Thilenius et al. ***(42)***, suggests that antibody agonists and natural ligand can stimulate different signaling pathways and emphasizes the limitations of defining physiologically important signaling pathways solely by antibody agonists.

3.12. Ceramides

Sphingomyelins are choline-headed phospholipids, ubiquitary components of eukaryotic cell membranes, whose function is unknown. In the course of apoptosis, their hydrolysis by sphingomylinases generates ceramide ***(43,44)***. Biologically active, cell-permeable, less-hydrophobic analogs of natural ceramides are powerful apoptotis agents. Many compounds of this family are available.

3.13. Matrix-Metalloproteases

Matrix metalloproteases (MMP) are a family that includes more than 20 members involved in extracellular matrix remodeling. They regulate endothelial cell proliferation, differentiation, and apoptosis (for review, *see* **ref. *45***). It seems that MT1-MMP (membrane type-MMP) and MMP2 (gelatinase-A), activated by MT1-MMP, play a pivotal role in the early steps of angiogenesis, controlling migration of endothelial cells in perivascular matrix and tubulogenesis. Highly specific antibodies against MMP are commercially available.

3.14. Poly-ADP-Ribose Polymerase (PARP)

PARP is a nuclear enzyme critical for DNA mechanism repair and is found ubiquitously in eukaryotic cells. PARP, activated in the presence of DNA strand breaks, synthesizes polymers of ADP-ribose that are added to nuclear protein acceptors such as istones, topoisomerases, and PARP itself. PARP is important as a target of cell-killing enzymes such as caspases in the early steps

of apoptosis ***(46)***. Monoclonal antibodies and synthetic inhibitors of PARP are commercially available.

4. Conclusions

This brief report describes the more up-to-date molecular mechanisms of genetically programmed cell death, as well as its deregulation in some hematopoietic malignancies. It is noteworthy that the deregulation of apoptosis could be either positive or negative in the different pathologies considered. The most widely used methods for the study of the apoptotic process are summarized and their possible applications in immunohematopoietic diseases are considered.

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16

Cytokine Receptors

Qualitative and Quantitative Analysis

Heddy Zola

1. Introduction

1.1. Value of Cytokine Receptor Analysis in Hematopoietic Malignancies

Much has been written on the possible role of cytokines in hematopoietic malignancies. In some instances, such as interleukin-6 (IL-6) in multiple myeloma, there is evidence for a central role in maintaining malignant growth ***(1)***. The relationship between IL-6 and multiple myeloma is probably more complex, involving other cytokines that signal through receptors, which share the gp130 chain with the IL-6 receptor. Another strong association is that of IL-2 with acute T-cell lymphoma/leukemia, which express and release large amounts of the IL-2 receptor-α chain (CD25, Tac) ***(2)***. In other hematopoietic malignancies there is no strong evidence for the involvement of cytokines in the malignant process. Nevertheless, if a malignant clone expresses receptors for a cytokine and that cytokine is available, the end result may be accelerated proliferation or enhanced survival of the malignant cells. Conversely, expression of receptor for cytokines such as transforming growth factor-β (TGF-β), which tend to inhibit proliferation, or molecules like Fas, which induce apoptosis, may control the malignant clone. Thus cytokine receptor expression may provide valuable information on the behavior of the malignant clone ***(3)***. These considerations make a case for screening of cytokine receptor expression as a part of routine diagnostic evaluation of patients with hematopoietic malignancy. It is emphasized, however, that at present cytokine receptor expression is a research tool, with the possible exception of the determination

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of soluble IL-2 receptor α chain (Tac, CD25). Measurement of soluble Tac has been widely reviewed *(4)* and will not be described here.

1.2. Cytokine Receptors: Structural Considerations

Cytokine receptors are often complex structures that are composed of two or more protein chains. In several instances, one protein confers cytokine-binding specificity, whereas a second protein may increase the binding affinity, is primarily involved in signal transduction, and may be shared by several other cytokines *(5,6)*.

1.3. Analytical Methods

Methods for analyzing cytokine receptor expression on cells can be classified as functional, ligand binding, or antibody based. Each approach has advantages and limitations.

1.3.1. Functional Methods

In the approach, cytokine is added to the cells and a response is looked for: do the cells proliferate, or exhibit changes characteristically induced by the cytokine in question? This approach is the most relevant to the behavior of the malignant cells because the mere expression of a receptor without functional consequence will not influence the disease. On the other hand, functional analysis without direct demonstration of receptor expression can be misleading, since we do not know whether the functional effect is direct or indirect. A cytokine may induce a contaminating normal cell population to secrete a different growth factor, which in turn may induce a functional response in the malignant population. Furthermore, functional methods based on incorporation of tritiated thymidine, for example, provide data on the population as a whole, and may conceal valuable information on subpopulations.

1.3.2. Ligand Binding Methods

These methods use the pure cytokine, usually radiolabelled, to measure binding by methods based on Scatchard analysis *(7)*. These techniques provide information on the number and affinity of binding sites with a precision and accuracy not available from other methods. The principal limitation is that they provide an average value for a cell population, without revealing subpopulations that may differ in cytokine receptor expression. The cytokine may be labeled with a fluorophore, or more commonly with biotin, which can be detected in turn with fluorophore-conjugated avidin. This approach, in common with the antibody-based methods described in **Subheading 1.3.3.**, allows analysis of a heterogeneous population on a cell-by-cell basis by flow

cytometry (FCM), or analysis of tissue distribution by immunohistological methods.

1.3.3. Antibody-Based Methods

Detecting cytokine receptors using monoclonal antibodies (mAbs) against the receptors is probably the easiest of the three approaches technically and the one most suited to diagnostic evaluation of individual patient samples, as it uses methodology widely available in diagnostic laboratories. There are two important limitations. First, an antibody will usually detect an individual receptor molecule. Hematopoietic growth factors generally use complex two- or three-chain receptors, and expression of an individual receptor does not confer responsiveness to the cytokine unless the rest of the receptor is expressed. The second limitation is that cytokines may activate cells through a very low concentration of receptor. This entails a requirement for sensitive analytical methods. Although the methods outlined so far depend on measuring function or expression of the receptor as protein, an alternative is to detect or measure mRNA for the receptor. Measuring receptor mRNA in bulk extracts will tell us nothing about individual cells and populations. If mRNA measurement is based on polymerase chain reaction (PCR) there is a risk that the mRNA detected may derive from a minor contaminating cell population because of the extreme sensitivity of PCR. *In situ* hybridization, especially flow cytometric fluorescence *in situ* hybridisation (Flow-FISH) allows cell-by-cell analysis, and may be attractive if an antireceptor antibody is not available. This potentially powerful technique has not received widespread use and is not described here.

1.4. Choosing the Appropriate Method

There is an enormous diversity of functional methods for the measurement of responses to cytokines. The most appropriate method depends on the cytokine and cell population in question. Functional methods are not described here, but the nature of the information obtained is discussed. Methods based on radioligand binding are highly specialized and would require a separate chapter. Methods that use fluorochrome-labeled ligands will be discussed briefly. The major emphasis will be on antibody-based methods, particularly techniques capable of providing the sensitivity necessary to detect receptors that may be present, and active, at concentrations as low as 100 molecules per cell. Methods will be described both for FCM and for microscopy. A major advantage in using FCM to analyze cytokine receptor expression is the ability to determine differential expression by subpopulations of cells, using multiparameter FCM. In the context of hematological malignancy, this usually means

a marker for the malignant population, although it may be of interest to assess cytokine receptor expression on, for example, T cells infiltrating a tumor.

1.4.1. Functional Methods

Cytokines characteristically have multiple functions. IL-6, for example, was originally known as B-cell differentiation factor ***(8)***, and was assayed by its ability to induce secretion of IgG. It is known to induce activation in T cells, proliferation of myeloma cells, and to act on non-hematopoietic tissues ***(8)***. Different cells respond differently to the same cytokine. Cytokines appear to act cooperatively or antagonistically in what has been referred to as the cytokine network. Thus, IL-4 and IL-5 may induce expression of IL-2 receptor chains ***(9)***; cytokines that share a common chain may compete for limited amounts of receptor. These interactions make it difficult to predict how a particular hematopoietic cell population, particularly the malignant expansion of a rare cell type, will respond to a cytokine. Descriptions of responses by hematopoietic cell populations to cytokines always require controls to show that it is indeed the malignant cell population responding. For example, if a chronic lymphoid leukemia (CLL) sample shows a proliferative response to IL-4, is the increased uptake of tritiated thymidine due to the malignant cells or to normal cells? Purifying CLL cells provides some reassurance, but it is difficult to obtain, and demonstrate, adequate purity. How many normal cells are needed to give a confounding response? A better approach is to use an assay that directly identifies the responding cells—to stay with the same example, demonstrating an increase in cells in the S phase of the cell cycle, in conjunction with a marker for the CLL cells, would be more convincing. CD19 may be a suitable marker, provided the fraction of cells in the S phase is large enough to ensure that the proliferating cells are not normal B cells. Membrane IgG may be better, in an IgG-positive CLL, as most circulating B cells are IgM positive.

Alternatively, showing that the proliferative cells are restricted to the light chain carried by the CLL cells would indicate that the normal B cells are not involved. If the response being tested is a change in phenotype, two-parameter phenotypic analysis may provide the answer. For example, a characteristic effect of IL-4 on B cells is upregulation of CD23 expression. Two-color FCM of CD23 with one of the markers discussed in the previous paragraph may provide convincing evidence that the CLL population is responding. As indicated in **Subheading 1.**, a further problem is that it is difficult to be sure that what is being measured in vitro is a direct effect of the cytokine on the malignant cells, as opposed to an indirect effect, mediated by a contaminating normal cell population. If the effect is one known to be associated with the cytokine (e.g., upregulation of CD23 by IL-4), this provides some

basis for confidence. However, it will always be difficult to rule out indirect effects. On balance, functional tests are most useful when the malignant cells have been shown to express the relevant receptors (by methods to be discussed shortly), to determine whether they are capable of responding ***(3)***. If the receptors are not expressed, there is limited value in looking for a response, although the caveat to that rationale is that the receptor may not be detected by direct means because of its low-level expression.

1.4.2. Radioligand Binding Methods

Radioligand methods have been critical in the development of an understanding of cytokine receptors. These methods demonstrated the presence of low- and high-affinity receptors for many cytokines, a finding that subsequently was understood in terms of the multichain receptors. In characterizing the cellular reactivity of a new cytokine, self-displacement analysis with radiolabeled cytokine forms an essential component. This analysis provides an indication of the binding affinity and number of receptor types (low affinity, high affinity), as well as the mean number of receptor molecules per cell. Once the receptors have been characterized, this type of analysis would be performed on new cell types, for example, malignant cells, only if there was a reason to suspect that the cells carry a different type of receptor. The type of receptor present can be more easily determined using mAbs against the receptor chains, whereas the number of receptor molecules per cell can also be obtained from fluorescence methods (*see* **Subheading 1.4.3.**).

1.4.3. Flow Cytometry Methods

Cytokine receptors can be expressed on in vitro activated cells at high concentrations; for example activation of human T cells with CD3 antibody leads to rapid up regulation of CD25 to >30,000 molecules per cell. However, receptors do not need to be at this level for cytokines to induce a functional response ***(10,11)***. Cells taken direct from tissue or blood commonly express concentrations around 100–500 molecules per cell ***(12,13)***. Conventional immunofluorescence/FCM is sensitive to 2000 molecules per cell, but specialized procedures can detect much lower levels ***(12,14)***. In the method described below, cytokine receptor protein is detected by mAb, which is detected with biotinylated antibody against the mouse immunoglobulin; the biotin is stained with phycoerythrin-streptavidin (PE-SA). This method can be combined with the detection of specific cell population markers (lineage, subset, activation markers) in multiparameter analysis. A variation uses biotinylated cytokine instead of mAb as the first step to study binding of the cytokine to its receptor. Common features of these assays are the use of PE as a highly effective fluo-

Table 1
Antibodies for Cytokine Receptor Proteins

Cytokine receptor	CD no.[a]	Supplier[b]
IL-1 type 1 (p80)	CD121a	Ph, Gen, RD
IL-1 type 2	CD121b	Gen, RD
IL-2Ra	CD25	Many
IL-2Rb	CD122	BD, Ph, IT, RD
IL-2Rg (common chain)	CD132	Ph, RD
IL-3R	CDw123	Ph, RD
IL-3/IL-5/GMCSF common	CDw131	Ph
IL-4R	CDw124	IT, Gen, RD
IL-5R	CDw125	Ph
IL-6R	CD126	Ser, Bio-S, RD
gp130(IL-6, LIF, IL11, etc.)	CD130	Ser
IL-7R	CD127	IT, Gen
TNFR p55	CD120a	Gen, RD
TNFR p75	CD120b	Gen, RD
IFNgamma R	CDw119	Gen, Ph
GMCSF	CD116	Ph
c-kit, scf-receptor	CD117	IT
FAS/APO-1	CD95	Ph
Ox40	CD134	Ph
Flt3, Flk2	CD135	—
MSP-R	CDw136	—
4-1BB	CDw137	—
IL-9R	—	RD
IL-10R	—	RD

[a]CD numbers allocated at the 5th and 6th International Workshops on Leucocyte Differentiation Antigens ***(20)***.

[b]Commercial suppliers: BD: Becton Dickinson; Bio-S: Bio-Source; Endo: Endogen; Gen: Genzyme; IT: Immunotech; Ph: PharMingen; Sero: Serotech; RD: R&D Systems.

rescent dye, multiple-layer staining to increase sensitivity, and selection of reagents for maximum sensitivity. More detailed descriptions may be found in ***(14)***.

2. Materials

2.1. Human Cytokine Receptor Detection with Antireceptor Antibodies

2.1.1. Reagents

1. MoAbs against many cytokine receptor proteins are shown in **Table 1**.
2. Biotinylated anti-mouse Ig is available from many suppliers, but the intensity of specific and nonspecific staining varies. Suitable reagents include those from

Vector Laboratories (cat. no. BA-2000) and Silenus Laboratories (special product, available on request, AMRAD, Melbourne).
3. PE-SA is available from several suppliers, but again staining intensity varies. We currently use PE-SA Sigma (cat. no. S3402) and the Caltag reagent is also very suitable.
4. PBS/azide: Dulbecco's phosphate-buffered saline (PBS), with sodium azide.
5. Normal horse serum; normal human serum.

3. Methods

3.1. FCM

3.1.1. mAb-Based Approach (*see* **Note 1**):

1. Maintain all stages at 0°C by placing reaction tubes in melting ice.
2. Prepare mononuclear cells from blood or tissue using Ficoll-hypaque. Suspend cells at 10^7/mL in PBS/azide. Identification of a particular cell type can be made by multiparameter staining and analysis. Whole blood may be used, lysing erythrocytes after staining and before analysis.
3. Add an optimized amount of mAb (*see* **Note 2**) to tubes (use tubes that hold about 3 mL and fit the flow cytometer sample uptake assembly).
4. Mix cell suspension and aliquot 50 µL per tube. Mix gently and incubate for 30 min, resuspending settled cells once after 15 min (*see* **Note 3**).
5. After incubation, add 3 mL of cold PBS/azide (stored in the refrigerator [*see* **Note 4**]) and centrifuge at 200 g for 5 min in a refrigerated centrifuge. Remove the supernatant by gentle aspiration, being careful to remove as much of the liquid as possible without disturbing the cell pellet (*see* **Note 5**). Resuspend the cells in 100 µL of cold PBS/azide, add 3 mL of cold PBS/azide, mix, and repeat the washing step.
6. Resuspend the cells thoroughly in 50 µL cold PBS azide and add 10 µL of normal horse serum and 5 µL normal human serum. Incubate at room temperature for 10 min, then return tube to ice water. The normal serum is added to block Fc receptors and thus reduce nonspecific binding of the Ig (*see* **Note 6**) which is used in the following step. If the anti-mouse reagent is made in a species other than the horse, use the appropriate species-blocking serum. Fc-mediated uptake can also be avoided by using F(ab)′2 preparations, but these reagents tend to be less sensitive.
7. Add 50 µL prediluted biotinylated anti-mouse Ig to the cells and mix gently. Incubate and wash as before.
8. Add 50 µL prediluted PE-SA to the cells and mix gently. Incubate and wash as before.
9. Resuspend cells thoroughly in 100–200 µL cold PBS/azide if FCM is to be carried out on the same day; if analysis is to be delayed, resuspend in FCM fixative ***(15)***. Adjust the volume used to give a data rate of about 100 cells/s on the cytometer.
10. Analyze by FCM. Select cell population of interest by gating on forward and side-scatter parameters and print out fluorescence intensity histograms (*see* **Fig. 1** for

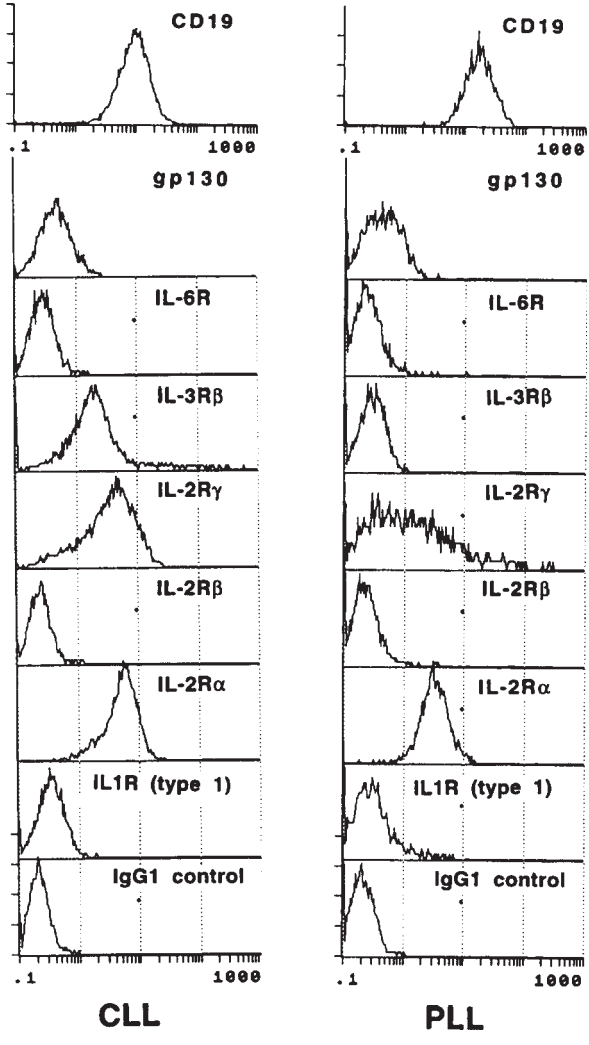

Fig. 1. Representative data for two leukemia samples tested for expression of a number of cytokine receptors. For additional examples, *see* **ref.** ***(3)***.

examples) (*see* **Notes 7** and **8**). Also refer to the FCM chapters in this volume for additional procedural and analytical details.

3.1.2. Labeled Cytokine-Based Approach (see Note 9*)*

1. Follow the procedure described in **Subheading 3.1.1.** for the antibody-based approach, except that the first layer is biotinylated cytokine and the second is PE-SA. Alternatively bound cytokine can be detected with anti-cytokine antibody ***(17)***.

2. Interpret samples immediately after staining and without the use of fixative, unless the cytokine/receptor complex is known to withstand fixation.

3.2. Fluorescence Microscopy (*see* Note 10)

3.2.1. Tissue Preparation and Staining

1. Perform all incubations at room temperature in a humidified chamber.
2. Cut fresh lymphoid tissue into blocks (5–10 mm^3), which are snap-frozen in optimal cutting temperature (OCT) compound (Miles Laboratories, Elkhart, IN). Frozen tissue blocks are stored at –80°C.
3. Place cryostat sections (6 µ) on gelatin-coated slides and air-dry overnight to ensure adherence to the slide.
4. Circle each section with a wax pen.
5. Cover each section with 3% bovine serum albumin (BSA) in Tris-buffered saline (TBS) for 30 min to block nonspecific binding of subsequent reagents.
6. Add mAb (100 µL) to the specimen, at a dilution in TBS/1% BSA shown by preliminary titration to give optimal staining with minimal background staining. This is usually a 10–20-fold lower concentration than used for flow cytometric analysis.
7. Wash with TBS containing 1% BSA, stirring for 5 min. Remove excess liquid by gentle blotting with paper tissue, immediately before the addition of the next reagent (*see* **Note 11**), being careful to avoid drying out.

3.2.2. Three-Stage Method (*see* Note 12)

Bound mAb is detected with biotinylated horse anti-mouse Ig (Vector Laboratories, Burlingame, CA, cat. no. BA-2000) (100 mL). This reagent requires absorption with tonsil cells to reduce nonspecific staining. Absorption is carried out with 10^8 fresh or cryopreserved tissue cells per milliliter of antibody at 1/100 dilution for 1 h on ice with occasional mixing. Tissue for absorption is similar to the tissue being studied, e.g., spleen for studies of spleen sections. The purpose of absorption is to remove antibodies in the conjugate that reacts directly with the tissue. Slides are incubated for 1 h and washed as described, and the bound biotin is then visualized using Cy3-SA (Jackson ImmunoResearch, West Grove, PA, cat. no. 016-160-084), 100 µL of a 1/500 dilution. Slides are again incubated and washed as before, after which excess buffer is removed by gently blotting with tissue paper. Slides are mounted in 90% glycerol in PBS. The Renaissance Tyramide Signal Amplification system (DuPont NEN) deposits biotin at sites of antibody binding, can be used in conjunction with Cy3-SA, and gives very good sensitivity. Cy3 is visualized using filter blocks supplied for rhodamine, such as the Leica N2.1 (excitation filter 515–560 nm, dichroic mirror at 580 nm, and barrier filter at 580 nm) or the Leica M2 (excitation filter 546 ± 14 nm, otherwise as N2.1). Immersion lenses with a high numerical aperture give the highest sensitivity.

4. Notes

1. To achieve maximum sensitivity, a number of factors must be optimized ***(12,14)***. The major factors are fluorochrome, staining protocol, and reagent quality. Phycoerythrin (PE) has a higher extinction coefficient (amount of incident light energy absorbed) and gives a better quantum yield (energy of emitted signal for a given absorbed energy) than other dyes that can be excited at available wavelengths. The staining protocol should optimize sensitivity, which means the separation between the signals from positive and negative cells. The choice of reagents is a major determinant of sensitivity. Most commercially available PE reagents are intended for use in two-color work where sensitivity is not required. Reagents must be screened for high-sensitivity work. If using an instrument with adjustable optics, optimize for PE sensitivity. The optimal concentrations of mAb, biotinylated anti-mouse Ig, and PE-SA must be titrated in a prior experiment. As a guide, if the antibody concentration is known, use 5 µg/mL; 50 µL of undiluted culture supernatant or 1/100 dilution of unpurified ascitic fluid is a good starting point. A 1/100 dilution of the Vector anti-mouse Ig and 1/20 of Sigma PE-SA are suitable. Retitrate each new batch or after prolonged storage (>1 mo). Note that the use of an isotype-matched negative control is essential, although not necessarily an adequate, control, because antibodies give different nonspecific binding for reasons such as aggregation or denaturation during purification or storage.
2. To titrate reagents, use three controls: an antibody that will clearly resolve positive and negative populations (e.g., CD3), an antibody that identifies positive cells adequately only if high-sensitivity reagents are used (e.g., CD25), and a negative control. A typical set of results is shown in **Fig. 2**, for human peripheral blood leukocytes (PBL). This analysis constitutes an essential quality control assay, to select reagents, to check activity after storage, and to check instrument performance. The CD25 pattern should give a clear population of positive cells.
3. Cells and reagents must be kept cold throughout the procedure to reduce loss of antigen and dissociation of the antibody–antigen complex. All reagents are stored refrigerated, and the reaction tubes are kept in contact with melting ice. Centrifugation is carried out at 4°C. The only exception is the blocking of Fc receptors, which is done at room temperature to increase the rate of binding.
4. Note that sodium azide can react with copper pipes to form an explosive compound. Check institutional safety regulations for disposal of azide-containing liquids.
5. After centrifugation, remove the supernatant thoroughly, to minize dilution of the next reagent. Be careful to avoid cell loss. In practice, some supernatant will be left behind, and if the amount left behind is not constant from tube to tube, the next reagent will be diluted to a variable degree, introducing variability in staining. Resuspending cells after each centrifugation should be done thoroughly to ensure a single-cell suspension.
6. The major sources of nonspecific staining are binding to Fc receptors and species crossreactivity of anti-Ig reagents. These reactions involve B cells and mono-

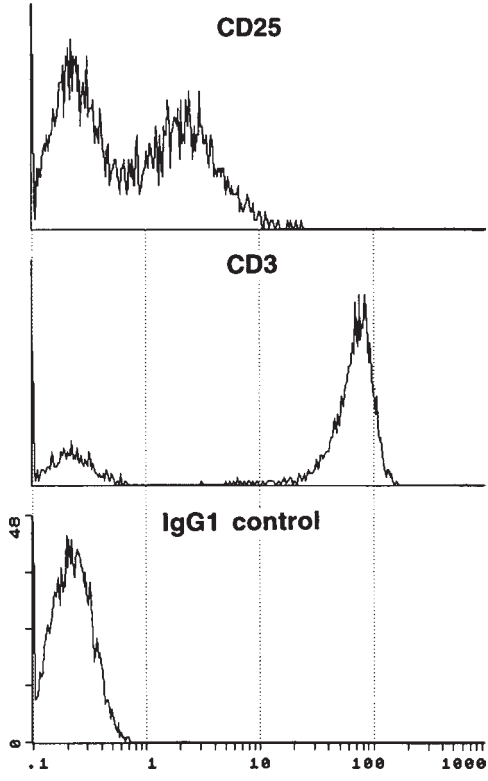

Fig. 2. Quality control for staining reagents and procedures. Fresh blood cells from a normal donor are stained with negative control, CD25, and CD3 antibodies, all IgG1, using the detection reagents described in the text. Results are examined for nonspecific staining (% positive and position of the negative peak with the negative control antibody); CD25 staining, which should give a bimodal distribution, with 30–50% positive without prior in vitro activation); and intensity of the CD3-positive peak.

cytes more than T cells. Crossreactivity of anti-mouse Ig with human Ig is a problem occasionally, even with reagents described as affinity purified or free of reactivity with human Ig, because of the higher sensitivity. Crossreactivity may be removed by absorption on immobilized human Ig, but it is preferable to use a batch that does not crossreact.

7. Analyze cell subsets using distinct fluorochrome-labeled mAbs. Fluorescein-conjugated or PE/Cy5-conjugated mAbs with specificities for lymphocyte subsets (CD3, CD4, CD8, CD19, etc.) and other cell populations are available in good quality from various suppliers (e.g., Caltag, PharMingen, Sigma, Becton Dickinson [who use PerCP as the third fluorochrome]). PerCP and PE/Cy5 energy transfer reagents (e.g., Tricolor from Caltag, CyChrome from Phar-

Mingen, Quantum Red from Sigma) are better than PE/Texas red combinations, because there is less spectral overlap with PE.
8. For simultaneous analysis of two molecules present at low concentrations, the dyes of choice are PE and the PE/Cy5 tandem system, which both have good extinction coefficients and quantum yields. The three-layer system can only be used for one of the two receptors being detected.
9. High-affinity cytokine receptor may be detected using biotinylated cytokine, which is detected in turn with PE-SA. In the case of multisubunit receptors, this will quantify the functional receptor, unlike mAb, which measures individual receptor chains. Human cytokines are available commercially from a number of companies (Genzyme, R&D Systems, PharMingen). If the cytokine is available biotinylated, it is best bought in that form; otherwise it can be biotinylated by standard methods ***(16)***.
10. Additional mAbs useful as controls or to identify particular regions of tissue, include anti-IgD, anti-IgM, CD19, CD3, and CD38.
11. Phycoerythrin gives the highest sensitivity in FCM but fades within seconds under microscope illumination. Cy-3 gives an intense red staining that appears to be as bright as PE staining in spite of its lower extinction coefficient and quantum yield.
12. Immunofluorescence staining of tissue sections can give sensitivity comparable to FCM for the detection cytokine receptors. For examples, see **refs.** ***18*** and ***19***.

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Hematologic Malignancies

Methods and Techniques

Edited by

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Recent advances in molecular biology are transforming the treatment of hematologic malignancies by providing powerful new tools useful in both the diagnosis and the assessment of new therapies. In *Hematologic Malignancies: Methods and Techniques,* a panel of acknowledged experts review many of the key molecular methods used for the diagnosis and subsequent management of hematologic malignancies. These clinically relevant techniques range from routine test procedures to highly sophisticated methods currently offered only by specialized reference laboratories, and fall into five major groups: cytogenetics, polymerase chain reaction, flow cytometry, cytochemistry and immunochemistry, and apoptosis and cytokine receptors. Presented by investigators with extensive experience, and serving not only as a reference for seasoned research and clinical laboratory personnel, but also as a guide for the less experienced, each method contains sufficient detail to ensure its successful replication.

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