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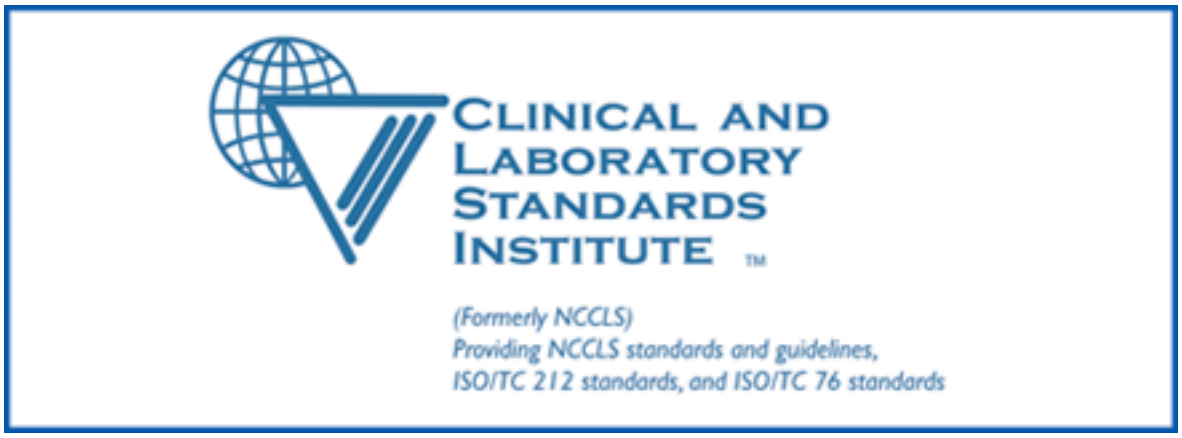
Immunoglobulin and T-Cell Receptor Gene Rearrangement Assays; Approved Guideline—Second Edition



This document provides guidance on the performance of gene rearrangement assays, including indications; specimen collection, transport, and processing; assessment of specimen adequacy; and quality control.

A guideline for global application developed through the NCCLS consensus process.

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Serving the World's Medical Science Community Through Voluntary Consensus

NCCLS is an international, interdisciplinary, nonprofit, standards-developing, and educational organization that promotes the development and use of voluntary consensus standards and guidelines within the healthcare community. It is recognized worldwide for the application of its unique consensus process in the development of standards and guidelines for patient testing and related healthcare issues. NCCLS is based on the principle that consensus is an effective and cost-effective way to improve patient testing and healthcare services.

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- the authorization of a project
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- the revision of documents in response to comments by users
- the acceptance of a document as a consensus standard or guideline.

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Healthcare professionals in all specialties are urged to volunteer for participation in NCCLS projects. Please contact the NCCLS Executive Offices for additional information on committee participation.

Immunoglobulin and T-Cell Receptor Gene Rearrangement Assays; Approved Guideline—Second Edition

Abstract

Assays that detect monoclonal rearrangement of immunoglobulin or T-cell receptor genes are useful adjunct methods in the diagnosis of leukemia or lymphoma. Prudent clinical use requires a thorough understanding of the sensitivity and technical artifacts associated with these methods, together with the ability to prudently weigh the results. NCCLS document MM2—*Immunoglobulin and T-Cell Receptor Gene Rearrangement Assays* helps laboratorians perform and interpret gene rearrangement assays. It includes indications for gene rearrangement analysis and acceptable methods for specimen collection, transport, and processing. Also included are recommendations for assessing specimen adequacy, as well as technical methods for conducting gene rearrangement assays, including information on sensitivity, specificity, controls, and test interpretation. Quality assurance procedures are included throughout the document.

NCCLS. *Immunoglobulin and T-Cell Receptor Gene Rearrangement Assays; Approved Guideline—Second Edition.* NCCLS document MM2-A2 (ISBN 1-56238-466-X). NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2002.

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Immunoglobulin and T-Cell Receptor Gene Rearrangement Assays; Approved Guideline—Second Edition

Volume 22 Number 12

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In addition, the area committee would also like to recognize the valuable contributions of the members the Subcommittee on Molecular Hematology that developed the first approved edition of this guideline.

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Contents

Contents (Continued)

Foreword

NCCLS document MM2-A2—*Immunoglobulin and T-Cell Receptor Gene Rearrangement Assays; Approved Guideline—Second Edition* is one in a series that address molecular methods technology. This guideline provides recommendations for performance and interpretation of molecular biologic assays in diagnostic hematopathology, specifically immunoglobulin and T-cell receptor gene rearrangement. This guideline addresses both technical methods and quality control for the performance of these two types of assays using nonamplification-based southern blot methods.

The methods and quality control approaches described herein are intended for use by both manufacturers and pathology laboratories. Such use is intended to facilitate both interlaboratory comparison of results and diagnostic interpretations, as well as to ensure accuracy in diagnosis.

This guideline is written for laboratory directors, surgical pathologists, hematopathologists, medical technologists, and manufacturers of instruments and reagents used in these assays.

Key Words

Gene rearrangement, hematopathology, leukemia, lymphoma, southern blot

The Quality System Approach

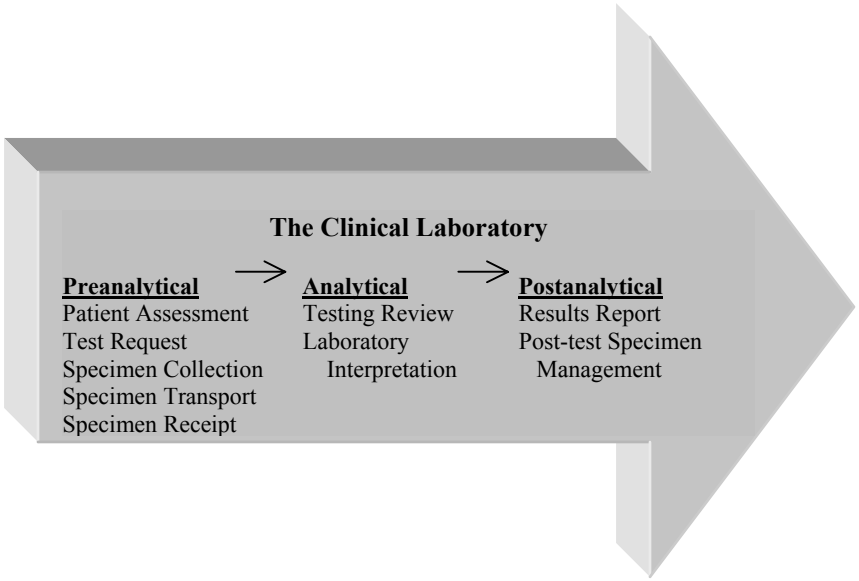
NCCLS subscribes to a quality system approach in the development of standards and guidelines, which facilitates project management; defines a document structure via a template; and provides a process to identify needed documents through a gap analysis. The approach is based on the model presented in the most current edition of NCCLS HS1- *A Quality System Model for Health Care.* The quality system approach applies a core set of "quality system essentials (QSEs)," basic to any organization, to all operations in any healthcare service's path of workflow. The QSEs provide the framework for delivery of any type of product or service, serving as a manager's guide. The quality system essentials (QSEs) are:

QSEs

Documents & Records	Information Management
Organization	Occurrence Management
Personnel	Assessment
Equipment	Process Improvement
Purchasing & Inventory	Service & Satisfaction
Process Control	Facilities & Safety

Path of Workflow

A path of workflow is the description of the necessary steps to deliver the particular product or service that the organization or entity provides. For example, NCCLS document GP26-A2 defines a clinical laboratory path of workflow that consists of three sequential processes: preanalytical, analytical, and postanalytical. All clinical laboratories follow these processes to deliver the laboratory's services, namely quality laboratory information. The arrow depicts the sequence, from left to right, that any clinical laboratory follows. In addition, the necessary steps or subprocesses are listed below them.



Adapted from NCCLS document HS1—*A Quality System Model for Health Care*

Most of NCCLS's documents relate to the clinical laboratory, so the most common path of workflow will be that depicted above. The path of workflow for other healthcare activities, e.g., respiratory services, imaging services, etc., or for other types of organizations, e.g., medical device manufacturers, will differ from that of the clinical laboratory. All such paths of workflow describe the sequence of activities necessary to produce the organization's or an entity's specific product or services. For those documents that relate to other paths of workflow, the icon will reflect different process steps.

MM2-A2 Addresses the Following Steps Within the Clinical Laboratory Path of Workflow

Preanalytical					**Analytical**		**Postanalytical**	
Patient Assessment	Test Request	Specimen Collection	Specimen Transport	Specimen Receipt	Testing Review	Laboratory Interpre-tation	Results Report	Post-test Specimen Management
X	X	X	X	X	X	X	X	X

Adapted from NCCLS document HS1—*A Quality System Model for Health Care*

Immunoglobulin and T-Cell Receptor Gene Rearrangement Assays; Approved Guideline—Second Edition

1 Introduction

The interpretation of biopsy and aspirate results, through which atypical lymphoid cells are identified, is often difficult. Malignant diseases can occasionally masquerade as benign processes, while reactive processes may simulate malignancies. The emergence of an understanding of normal and abnormal development of the lymphoid system has enabled development of immunological and molecular markers for the identification of monoclonal populations of lymphocytes; identification of such a monoclonal population can significantly assist the diagnostician in arriving at the diagnosis of leukemia or lymphoma.[1-7]

Prudent clinical use of the ability to identify monoclonal proliferations of lymphoid cells using molecular methods requires a thorough understanding of the sensitivity and technical artifacts associated with these methods. Avoiding erroneous interpretation of B- and T-cell gene rearrangement assays requires careful attention to technical detail and the use of rigorous quality assurance measures.[8,9] This guideline helps laboratorians that rely on gene rearrangement assays to perform these techniques using the appropriate controls. The guideline also helps laboratorians decide what types of materials and records are to be kept following the laboratory procedure, as well as the length of time each is to be kept. Finally, the guideline helps both manufacturers of diagnostic kits and reagents, and those responsible for monitoring compliance with quality assurance programs.

2 Scope

The use of molecular methods that use deoxyribonucleic acid (DNA) probes in clinical diagnosis presents new challenges to the pathologist. Despite the clear benefits of having another method by which to identify proliferation of monoclonal cell populations, issues of sensitivity and false-positive results mandate the application of stringent laboratory practice. To ensure the success of nucleic acid diagnostics, several key areas warrant attention. This document addresses the following topics as they relate to the direct detection of T- and B-cell gene rearrangements:

- indications for gene rearrangement analysis;
- specimen collection, transport, and processing;
- assessment of specimen adequacy;
- conduct of the gene rearrangement assay;
- sensitivity, specificity, controls, and artifacts;
- quality assurance; and
- interpretation of results.

3 Standard Precautions

Because it is often impossible to know what might be infectious, all human blood specimens are to be treated as infectious and handled according to "standard precautions." Standard precautions are new guidelines that combine the major features of "universal precautions and body substance isolation"

practices. Standard precautions cover the transmission of any pathogen and thus are more comprehensive than universal precautions which are intended to apply only to transmission of blood-borne pathogens. Standard precaution and universal precaution guidelines are available from the U.S. Centers for Disease Control and Prevention (*Guideline for Isolation Precautions in Hospitals*. Infection Control and Hospital Epidemiology. CDC. 1996;Vol 17;1:53-80), (MMWR 1987;36[suppl 2S]2S-18S), and (MMWR 1988;37:377-382, 387-388). For specific precautions for preventing the laboratory transmission of blood-borne infection from laboratory instruments and materials and for recommendations for the management of blood-borne exposure, refer to the most current edition of NCCLS document M29—*Protection of Laboratory Workers from Occupationally Acquired Infections.*

4 Definitions[a]

Absorbance, A, *n* - ***In optics,*** the capacity of a substance to absorb radiation. **NOTE:** Expressed as the logarithm of the reciprocal of the transmittance of the substance*;* $A = \log (1/T) = -\log (T)$.

Accuracy, *n* - Closeness of the agreement between the result of a measurement and a true value of the measurand {/analyte}*;* **NOTES:** a) Usually expressed in the same units as the result, as the difference between the true value and the value, or as a percentage of the true value that the difference represents; expressed this way, the quantity is more correctly termed "inaccuracy"; b) "Accepted reference value" may be used in place of "true value"*;* c) The difference includes contributions not only from process inaccuracy but also from process imprecision, especially when one determination per specimen is the rule; d) The relevant meaning of the term "accuracy" from the patient's point of view.

Agarose, *n* - A carbohydrate material used for preparing gels for the electrophoresis step used in southern blotting.

Annealing, *n* - The hybridization of two complementary strands of DNA or ribonucleic acid (RNA), as in the hybridization of a probe with the target DNA.

Atypical, *adj* - A term used by pathologists to describe cells or tissues having some or all of the morphologic characteristics associated with malignancy.

Biotin, *n* - A molecule that can be covalently attached to lysine residues of proteins; **NOTE:** This property is used in many detection systems.

Buffer, *n* - A solution or reagent that can resist a change in pH upon addition of either an acid or a base.

Chromosome, *n* - A single, large DNA molecule with its associated proteins that contains many genes and functions to store and transmit genetic information.

Control//Control material, *n* - A device, solution, or lyophilized preparation intended for use in the quality control process. **NOTES:** a) The expected reaction or concentration of analytes of interest are known within limits ascertained during preparation and confirmed in use; b) Control materials are generally not used for calibration in the same process in which they are used as controls.

Cryoquick, *n* - Any material used to embed tissue for frozen sections.

Definitive method (DM), *n* - An analytical method that has been subjected to thorough investigation and evaluation for sources of inaccuracy, including nonspecificity; **NOTES:** a) The magnitude of the DM's

[a] Some of these definitions are found in NCCLS document NRSCL8—*Terminology and Definitions for Use in NCCLS Documents.* For complete definitions and detailed source information, please refer to the most current edition of that document.

final imprecision and bias, expressed in the uncertainty statement, is compatible with the DM's stated end purpose; b) The mean DM value is taken as the "true" value.

Denaturation, *n* - Loss of native structure or configuration of a macromolecule, usually with resulting loss of biological or immunological reactivity or solubility; **NOTES:** a) In this document, denaturation is the conversion of double-stranded DNA or RNA to a single-stranded state with minimal secondary structure; this is done by heating, increasing the pH, or adding agents, such as formamide or urea; b) Once they are denatured, nucleic acid molecules are available for hybridization with a primer or probe.

Deoxyribonucleic acid (DNA), *n* - A type of nucleic acid; a polynucleotide having a specific sequence of deoxyribonucleotide units (dNTPs) and serving as the carrier of genetic information.

Diagnostic test, *n* - A measurement or examination of a diagnostic specimen for the purpose of diagnosis, prevention, or treatment of any disease or the assessment of health or impairment of health of an individual patient.

Ethylene diamine tetraacetic acid (EDTA), *n* **-** One of a class of aminopolycarboxylic acids that act as sequestering (also referred to as "chelating") agents; **NOTES:** a) They form soluble complexes with metal ions, removing these ions from further reactions; b) They are negatively charged compounds.

Equivocal result*, n* - A test result within a specified range of the cut-off value that cannot be interpreted as either positive or negative.

Gel electrophoresis, *n* – A process that causes separation of molecules in an electric field within a matrix of agarose or polyacrylamide according to size and charge.

Gene, *n* - A chromosomal segment that codes for a single polypeptide chain.

Gene rearrangement, *n* - The normal process by which immunoglobulin genes are assembled into DNA sequences capable of coding for immunoglobulin or T-cell receptor genes; **NOTE:** Gene rearrangement occurs as a normal part of the developmental maturation of B- and T-lymphocytes.

(Gold standard), *n* - A nonspecific term that indicates that a process or material(s) is the best available approximation of the truth. Its use is deprecated (See "definitive method" and "reference method.")

Hybridization, *n* - A base pairing of complementary strands of nucleic acid by hydrogen-bond formation; the binding of probe to specific nucleic acid sequences or amplification products; **NOTE:** Hybridization can be performed with both nucleic acid target and probe in solution, or with the nucleic acid target retained within a tissue specimen; this latter form of hybridization is referred to as "*in situ* hybridization."

Inaccuracy, *n* - The numerical difference between a value and the true value.

Leukemia, *n* - A type of malignant neoplasm ("cancer") that predominantly affects circulating blood cells and the bone marrow.

Lymphoma, *n* - A type of malignant neoplasm ("cancer") that predominantly affects cells in solid lymphatic tissue, such as lymph nodes.

Matrix, *n* - All components of a material system, except the analyte; **NOTE**: The effect (i.e., interference) of the matrix on an analytical method is commonly referred to as a "matrix effect."

Monoclonal, *adj* - Arising from a single clone of cells; **NOTE:** a) In this document, a population of lymphoid cells is considered to be monoclonal if each cell within that population harbors immunoglobulin or T-cell receptor genes that have the same rearrangement as all other cells within that population; b) Normal or reactive populations of lymphoid cells display small and approximately equal populations of many cells showing many different gene rearrangements; c) Demonstration that a large proportion (greater than 1%) of the cells within a lymphoid cell population have the same gene rearrangement pattern is often strong evidence that this population is malignant.

Nucleic acid, *n* - A polynucleotide made up of dNTPs (DNA) and rNTPs (RNA) linked to one another by phosphodiester bonds, forming macromolecular structures.

Phosphorimager, *n* - A device used to record the amount and intensity of a radioactive signal on a blotting membrane; **NOTE:** A phosphorimager may be used to replace film autoradiography.

Probe, *n* **-** A defined piece of single-stranded nucleic acid used to identify specific DNA or RNA molecules bearing the complementary sequence; **NOTE:** A probe can carry a label (radioactive or chemical) so that the probe can later be detected.

Reference method (RM), *n* **-** A thoroughly investigated method, in which exact and clear descriptions of the necessary conditions and procedures are given for the accurate determination of one or more property values, and in which documented accuracy and precision of the method are commensurate with the method's use for assessing the accuracy of other methods for measuring the same property values, or for assigning reference method values to reference materials*;* **NOTE:** Several categories of reference method exist, including: **Class A reference method,** which is characterized by both sufficient accuracy and precision and by a low incidence of susceptibility to known interferences, so that the stated purpose of the analytical process can be achieved, all of which is demonstrated by direct comparison with the definitive method; **Class B reference method,** which is believed to be of the caliber of a Class A Reference Method, except that the process of evaluation with a definitive method and a certified reference material has not yet been completed; **Class C reference method,** which is believed to be of the caliber of a Class A Reference Method, except that no definitive method is likely to become available.

Sequence, *n* - The order of nucleotides (A, C, G, T, or U) in a stretch of DNA or RNA.

Signal, *n* - A quantity that represents the measurand and which is functionally related to it; **NOTE:** A signal may be characterized by chemical, radioactive, luminescent, or colorimetric output.

Solid phase, *n* - One of several support media to which either target or probe nucleic acids are immobilized; **NOTE:** Examples include nylon or nitrocellulose membrane, beads, magnetic particles, and microtiter plate wells.

Southern blot, *n* - A solid phase membrane to which DNA, which was transferred from a gel after electrophoresis, is bound so that it can be hybridized with a labeled nucleic acid probe.

Stringency, *n* - The degree of specificity in a DNA hybridization or annealing reaction; **NOTES:** a) Stringency is adjusted by altering the salt concentrations in the buffer and/or adjusting the temperature of the reaction; for example, increasing the salt concentration decreases the stringency (more DNA will bind less specifically, allowing for more base-pair mismatches to occur); higher temperature increases the stringency (less DNA will bind more specifically); b) Stringency considerations apply to all annealing steps in amplification, as well as hybridization and wash conditions in detection; conditions can be designed to allow only perfect hybridization, or to tolerate a certain degree of mismatch.

Target, *n* - The area of the nucleic acid to be detected or amplified for detection (also called "template").

Template, *n* - See **Target.**

Transcription, *n* - The process of making RNA from DNA.

5 Indications for Gene Rearrangement Studies

Gene rearrangement studies are expensive, technically demanding, and time-consuming procedures, the use of which should be properly indicated if they are performed for patient care. Other diagnostic tests, including histologic, cytochemical, immunologic, cytogenetic, and, occasionally, other studies on patient specimens, frequently diminish or obviate the need for gene rearrangement studies. The results of gene rearrangement tests would be interpreted in consideration of these studies and clinical circumstances. Within these general guidelines, the following are appropriate uses of gene rearrangement studies using immunoglobulin and T-cell antigen receptor gene probes:

- to distinguish a reactive (frequently, histologically "atypical") from a clonal lymphoproliferative process in tissue or blood;
- to document organ involvement by a lymphoproliferative disorder (e.g., skin);
- to facilitate distinction between poorly differentiated carcinoma and "anaplastic" large-cell lymphoma;
- to detect small amounts of residual or recurrent lymphoproliferative disease in a tissue or body fluid specimen [particularly if prior gene rearrangement studies have been done as a guide to what band(s) the laboratory should look for];
- as a general aid in the distinction of acute lymphocytic leukemia (ALL) from acute myelogenous leukemia (AML);
- as an indicator of B- or T-lymphoid origin for a histologically neoplastic proliferation of lymphocytes, particularly if immunologic studies are not available or are inconclusive[9];
- as an aid in the distinction of Hodgkin's disease versus non-Hodgkin's lymphoma; and
- to determine whether two neoplastic biopsies reflect two different disease processes.

The determination that a gene rearrangement assay will be clinically useful in a given case generally is made by the pathologist, often in consultation with the attending physician.

6 Specimen Identification and Accessioning

6.1 Specimen Identification

The container in which each specimen is received should be clearly marked with a unique patient identifier, such as a hospital patient identification number. In most situations, the patient's name is not sufficient, although the combination of patient name and birth date is generally enough to prevent identification errors. The container should also be labeled with the date and time that the specimen was acquired.

6.2 Requisition Forms

All specimens should be accompanied by a requisition form that contains, at a minimum, the following information:

- patient name;
- date of birth;
- sex of patient;
- unique identifier found on the specimen container;
- site from which specimen was obtained;
- condition in which specimen was submitted (fresh, frozen, or ethanol-fixed);
- reason for requesting the test; and
- referring physician or health professional.

6.3 Criteria for Rejecting Specimens

Each laboratory should have written criteria for the acceptance or rejection of specimens. Rejection of specimens is strongly recommended if either the specimen or the requisition form lacks sufficient information for the laboratory or clinician to uniquely identify the specimen, or lacks other information necessary to determine if the specimen or test requested is likely to be unsuited to answer clinical questions. Specimen rejection may also be justified on the grounds of improper handling or transportation of the specimen, or submission of an inappropriate specimen.

Other conditions for accepting or rejecting specimens are left to the discretion of the laboratory. For example, some laboratories might choose not to accept formalin-fixed, paraffin-embedded material because of the diagnostic uncertainty associated with gene rearrangement assays performed on DNA extracted from such material. Other laboratories might choose to use these specimens, because finding clear evidence of gene rearrangement can be meaningful in individual cases. If a laboratory accepts specimens that are of limited utility as a result of the condition in which they are accepted, the final report should indicate the potential limitations of the assay imposed by the condition of the specimen.

Under certain conditions, use of nonoptimal specimens is justified on clinical grounds. For example, one cannot obtain a splenectomy specimen twice from the same patient. Therefore, as with other clinical laboratory procedures, the ultimate criterion on which to determine whether to perform a gene rearrangement assay must be based on the best interests of the individual patient.

6.4 Accessioning Specimens

Assign a unique laboratory identifier to each specimen when accepted for testing. This identifier must be linked with the unique patient identifier and with other identifiers, such as those for individual gels that may be used by the laboratory. The unique laboratory identifier should differentiate both among specimens from different patients and among different specimens taken from the same patient.

7 Recordkeeping, Record Retention, and Specimen Retention

7.1 Types of Records

Molecular diagnostic procedures differ from most other laboratory tests in that assays involving material from several different patients may be placed on a single gel for electrophoresis and subsequent blotting. The southern blots produced from these gels may be recorded using any of several different media, including:

- membranes containing bands that are visualized by colorimetric techniques, such as immunoenzyme methods;
- x-ray film containing autoradiographs of the blot;
- photographic film containing images of bands that are visualized by chemiluminescent assays; and
- computer records generated by devices such as phosphorimagers (hard copies of such records may also be available).

In addition, photographs of ethidium bromide-stained gels are frequently available.

7.2 Identification and Cross-Linking of Records

All records of the type described in Section 7.1 should be permanently labeled with a unique assay identifier that can be linked to the unique laboratory identifier of each specimen and with the unique patient identifier. An assay record should accompany each such permanent record and be uniquely linked to it. This assay record should record the nature of the test represented by the gel (such as "kappa chain rearrangement, *Bam*HI digest, probed with J_κ probe having a specific activity of <activity> on <date>"). The individual assay record should also include information on controls for that assay. Interpretation of control data is facilitated by including control assays on all gels and blots used for patient specimen assays.

Linkage of records may be carried out via logbooks, card files, or computer databases. However, it should be possible to completely review the assay conditions and results for any individual case using the chosen method of record linkage and to review any one of the unique identifiers.

7.3 Retention of Patient Materials and Records

All reports should be retained by the laboratory for a period of at least ten years, as should all records pertaining to the conduct of the assay and any computer records or images that record the assay results (e.g., autoradiographs of southern blots).

DNA extracted from patient specimens should be retained by the laboratory for at least ten years or whatever period is specified by state or federal regulations, or by laboratory procedures for the retention of surgical pathology specimens.

8 Specimen Transport and Storage

There is limited data on the absolute requirements for transport and storage of tissues for gene rearrangements. The following recommendations are based on commonly accepted practices.

8.1 Transport and Storage of Solid Tissue

Solid tissue from *post mortem* or surgical specimens is appropriate for gene rearrangements. Solid tissue can be fresh, frozen, and powdered followed by freezing, or frozen as a small chunk. If not processed within 24 hours, tissue should be frozen or fixed immediately after removal.

8.1.1 Frozen Tissue

Snap-frozen tissue can be transported on dry ice and stored at –70 ± 10 °C.

8.1.2 Fresh Tissue

Fresh tissue can be transported on wet ice. Thinly sliced (less than 0.5 cm) tissue can be kept in cold phosphate-buffered saline (PBS) at 2 to 6 °C for up to 24 hours.

8.1.3 Fixed Tissue

Solid tissue minced in ethanol can be transported and stored at room temperature (22 to 25 °C) for periods greater than one year. Solid tissue should be fixed immediately after removal from the patient.

8.2 Transport and Storage of Thin Sections

Thin sections from paraffin-embedded tissue or frozen blocks are appropriate for gene rearrangements, although paraffin-embedded tissues are suboptimal and are frequently technically unsatisfactory. Tissue for frozen sections should be frozen immediately after removal. Tissue for paraffin sections should be fixed immediately after removal.

8.2.1 Frozen Sections

Thin sections cut in a cryostat should be kept frozen until processing. Frozen sections can be transported when packed in dry ice, and they can be stored at –70 ± 10 °C.

8.2.2 Fixed Sections

Fixed, paraffin-embedded tissue can be stored at room temperature (22 to 25 °C) for more than five years. Extremes of temperature and periods of storage longer than five years can decrease the quality of DNA retrieved.

8.3 Transport and Storage of Cells and Other Fluids

Cultured cell lines; cells from cerebrospinal, pleural, pericardial, and ascitic fluids; as well as isolated mononuclear cells from blood and bone marrow are appropriate for gene rearrangement studies. Due to volume considerations, cells are usually pelleted by centrifugation and frozen or reconstituted in a minimal volume for freezing or transport. Cells can be received fresh, frozen in cryopreservation media, frozen as dry pellets, or viably frozen in dimethylsulfoxide (DMSO).

8.3.1 Frozen Cells

Cell pellets and frozen cell suspensions should be stored at -70 ± 10 °C. Pellets and frozen cell suspensions can be transported when packed in dry ice.

8.3.2 Fresh Cells

Cell pellets and fresh cell suspensions can be stored at 2 to 6 °C for brief periods of time. For 24-hour storage, cell suspensions that include fetal calf serum in buffered cell culture media are preferable to cell pellets. Cell pellets can be transported on wet ice for same-day receipt. Cell suspensions can be transported on wet ice for overnight delivery.

8.4 Transport and Storage of Whole Blood and Bone Marrow Aspirates

8.4.1 Whole Blood

Anticoagulated whole blood can be stored at room temperature (22 to 25 °C) for 24 hours, and it can be stored at 2 to 6 °C for up to 72 hours. Anticoagulated blood can be transported overnight at room temperature (22 to 25 °C). As a specimen ages, the quality of its DNA becomes increasingly poorer, which can cause results to be uninterpretable. Use of heparin anticoagulants may impair certain restriction endonucleases.[10] Nevertheless, heparinized specimens usually give acceptable results.

8.4.2 Bone Marrow Aspirates

Anticoagulated bone marrow aspirate is less stable than whole blood at room temperature (22 to 25 °C), and it should be stored and transported at 2 to 6 °C. However, adequate results are frequently obtained with aspirates stored and transported at room temperature (22 to 25 °C) for up to 24 hours. To prevent clotting, anticoagulants should be added. Acceptable anticoagulants include sodium heparin (2,000 U/mL or greater) and ethylene diamine tetraacetic acid (EDTA) (standard lavender-top tube). As a specimen ages, the quality of its DNA becomes increasingly poorer, which can cause results to be uninterpretable.

9 Quality Control

9.1 Reagent Quality Control

Because of the large number of reagents required for complex procedures, and that many of the reagents must be prepared in the laboratory rather than purchased premixed, quality control is especially important in the molecular hematology laboratory. In general, complex buffers and solutions should be prepared in advance and given a batch number. This batch number should be recorded on the reagent preparation form and also on the reagent label. Information on all reagent lot numbers, expiration dates, amounts required, and balance/pipet calibration dates should also be recorded. New reagents should then be tested comparatively with the previous batch of the same reagent to ensure that acceptable results are achieved. Records of the dates of preparation and test results of any prepared reagents, along with lot numbers and their dates of use, should be kept in a logbook. Reagent bottles should be labeled clearly with the contents, lot numbers, and expiration dates. The expiration dates of certain reagents that do not expire, such as sterile water, may be listed as "undefined" or "not applicable."

9.2 Procedural Quality Control

To ascertain whether the results obtained from a gene rearrangement assay are valid patient management tools, a number of procedural quality control measures are required. These controls include (in addition to running the usual positive and negative controls) additional assays to determine the quantity and quality of DNA used in a given assay, the specific activity of the probes used, and a variety of other controls. These quality control measures are detailed in the procedure outline that appears in Section 10.

For each gene rearrangement assay performed in the laboratory, a procedural quality assurance record should be generated and maintained. Information that should be recorded includes:

- an indication that the specimen and requisition form were examined and criteria for specimen acceptance met (Sections 6.1 to 6.3);

- the condition of the specimen upon receipt (Section 8);

- any decision to accept a specimen for testing despite a failure to meet the written acceptance criteria (such a decision should be "signed off" by the laboratory director and a notation made as to the reason the specimen was accepted [Sections 6 and 8]);

- the DNA quality and yield (Section 10.4);

- the quantity of DNA used in the restriction digests, if different than that called for in the procedure manual (Section 10.6);

- the concentration of agarose and voltage/duration of electrophoresis, if different than that called for in the procedure manual (Section 10.7.3);

- the results of DNA visualization (Section 10.7.4);

- specific activity/concentrations of probes, if appropriate (Section 10.8.3);

- autoradiography conditions, if different than called for in the procedure manual (Section 10.9.4); and

- evidence that positive and negative controls were examined and found to be within expected limits (if results are reported when this condition is not met, a rationale for reporting the results should be recorded and also provided with the final report).

9.3 Equipment Maintenance

All equipment used in these procedures should be maintained and calibrated according to the manufacturer's standards and other applicable NCCLS standards and guidelines. All balances, pipets, spectrophotometers, gel electrophoresis power supplies, and incubators should be calibrated on a regular cycle.

9.4 Quality Assurance Procedures and Records

9.4.1 Procedure Manual

A procedure manual should be available within the laboratory that details every aspect of laboratory performance and specifies the quality assurance procedures and recordkeeping used within the laboratory. Detailed specifications for reagent quality control records (Section 9.1), procedural quality control records (Section 9.2), and equipment quality control records (Section 9.3) should be defined.

9.4.2 Personnel Standards

The procedure manual or a separate personnel manual should indicate the level of education, training, and experience required for personnel who perform any of the assay procedures in the laboratory. Special care should be taken to define the qualification requirements for personnel responsible for review of the final laboratory test results and release of these results from the laboratory (“sign-out”). The personnel standards should define the location in which personnel qualification statements are kept, and it should define methods by which personnel qualification reviews/performance reviews are documented.

10 Immunoglobulin and T-Cell Receptor Gene Rearrangement Assay Procedures

10.1 Procedure Overview

Traditional assays for immunoglobulin and T-cell receptor gene rearrangement consist of the following steps, which are outlined in greater detail in later sections.

(1) Isolate DNA from the appropriate cells.[11,12]

(2) Digest genomic DNA with the appropriate restriction enzymes.[11,12]

(3) Fractionate the digested genomic DNA on agarose gels and perform a southern transfer.[11,12,13]

(4) Radiolabel the appropriate probe. [11,12,14]

(5) Hybridize the appropriate radiolabeled probe to the southern membrane.

(6) Perform autoradiography and detect the changes in the appropriate gene structure.

10.2 Technical Limitations

Southern blot assays are highly effective in demonstrating a clonal population when 10% or more of the sample contains a clonal rearrangement. The southern blot test for changes in gene structure requires that, minimally, 1 to 5% of the input cells or tissue are potentially malignant or potentially contain the rearranged or translocated genes. If the pathologist estimates that greater than 1% of the input cells are representative of the disease state, then it is possible that gene rearrangement will help obtain valuable information needed to characterize a biopsied specimen. If less than 1% of the input cells are representative of the disease state, then gene rearrangement assays are significantly less likely to contribute to patient management.[1-8,15-18]

10.3 DNA Isolation

DNA extraction requires the lysis of cells, digestion of protein, removal of RNA, and extraction followed by precipitation of DNA.

10.3.1 Quantity of Tissue Required

The amount of DNA required depends on the precise protocol in use within the laboratory. For example, if one uses three probes (J_H, J_λ, and TCR_β), three restriction enzymes per probe, and 5 µg DNA per lane, then approximately 50 µg of DNA is required. Thus, extraction of greater than 100 µg of DNA provides an excess of DNA, which allows for potential repetitions of the assays as needed. Preferably 2 x 10^8 nucleated cells, or 500 mg of tissue, is sufficient to obtain greater than 100 µg of purified DNA. For laboratories that use larger numbers of restriction digests or load more DNA onto the agarose gel, these numbers may be scaled as required.[8]

10.3.2 Preparation of Solid Tissue Chunks

Solid tissue can either be used immediately after surgery or *post mortem*, or it can be snap-frozen and stored at -70 ± 10 °C for later extraction. To snap-freeze a piece of tissue, it should be dropped into liquid nitrogen. The amount of tissue sufficient to obtain greater than 100 µg of purified DNA is 500 mg.

Frozen tissue can be ground with a mortar and pestle, adding liquid nitrogen as necessary to keep tissue frozen. The frozen powdered tissue can be added to the lysis solution, then vortexed rapidly. Thawed or fresh tissue (especially small quantities) can be minced in lysis buffer with sterile scalpels or scissors.

10.3.3 Preparation of Thin Sections of Solid Tissue

Solid tissue that has been cryopreserved for frozen section immunohistochemical analysis can be used for DNA isolations. The frozen cryoquick-covered tissue can be sliced into the appropriate number of 20-µm sections to determine the weight of tissue necessary to yield a specific amount of DNA, as indicated by the formula that follows. The cryoquick preservative must be removed from the tissue sections. The person cutting the sections removes the surrounding cryoquick and transfers the frozen sections to a prechilled (-70 ± 10 °C) and preweighed microcentrifuge tube. A formula for the number of 20-µm sections needed to obtain approximately 100 µg of DNA is as follows:

$$\#20-\mu m\ Sections = \frac{1}{Surface\ area\ in\ cm^2} \cdot \frac{100\%}{\%\ Cellularity\ Relative\ to\ Lymph\ node\ or\ Tonsil} \cdot 6.7$$

The tube containing the sections is transported on liquid nitrogen to the analytical calibrated balance for weight determination. After weighing, the tube can be transported on liquid nitrogen to the -70 ± 10 °C freezer for storage as solid tissue for later DNA isolation. The lysis volumes used will be proportional to the mass of tissue for lymph nodes and other very cellular tissues.

10.3.4 Preparation of Cells and Other Fluids

Cultured or primary cells should be pelleted out of the media, and can be washed once in sterile PBS. The amount of nucleated cells sufficient to obtain greater than 100 µg of purified DNA is 2 x 10^8 cells.

Pellet cells gently at approximately 300 to 400 × *g* for seven to ten minutes and remove all fluid without disturbing the cell pellet. Now the cell pellet can be lysed, or the pellet can be quick frozen in liquid nitrogen or in a -80 °C cryobath and stored in the freezer at -70 ± 10 °C for later lysis. Cells can be viably frozen in DMSO and stored in the -70 ± 10 °C freezer for later lysis.

10.3.5 Preparation of Whole Blood, Bone Marrow, Buffy Coat, or Leukophoresis Product

The amount of mononuclear cells sufficient to obtain greater than 100 µg of purified DNA is 2 x 10^8. The quantity of specimen necessary to obtain this final number of cells depends on number of cells per milliliter. If the white count is within normal limits, one can use 3 to 10 mL of whole blood or 1 to 2 mL of bone marrow in EDTA, heparin, or acid citrate dextrose (ACD) to get this final number of cells.

Mononuclear cells are usually isolated by density gradient separation before DNA isolation.

10.3.5.1 Density Gradient Separation of Mononuclear Cells

Whole blood or bone marrow diluted with PBS is layered on the surface with an equal volume of room-temperature, density gradient solution in 50-mL tubes. The extent of dilution is dependent upon the density gradient system used. Tube(s) are centrifuged gently at approximately 400 × *g* for 30 minutes at 16 to 20 °C. The buffy coat is removed and placed in a 50-mL tube; samples should be pooled if greater than one gradient is required. Resuspend the white cells by pipetting up and down in PBS. Bring the PBS up to 50 mL and centrifuge at low speed (typically around 100 rpm). Decant the supernatant and add 5 to 29 mL of PBS, depending on the size of the pellet. Count the cells and remove 3 to 5 x 10^7 cells for DNA analysis.

DNA can also be isolated from whole blood directly, but the presence of the heme group can interfere with some tests.

10.3.6 Lysis of Tissue and Degradation of RNA and Protein

Once the material to be extracted is defined (cells versus tissue, mass or cell number, relative cellularity of tissue, fresh versus frozen, frozen chunk versus frozen 20-µm sections, etc.), the necessary lysis volume and type of mixing can be determined.

The type of mixing to be used is determined by the characteristics of the material and its storage conditions. Avoid cross-contamination between specimens, and use disposable supplies. The mortar and pestle can be used on frozen chunks of tissue. Mincing with a disposable scalpel or razor blade is appropriate for small specimens. Vigorous vortexing can be used on fresh and frozen cell pellets, or on frozen 20-µm sections.

Determine the appropriate lysis volume based on the type and size of the specimen. With standard lysis buffers, the following are typical lysis volumes:

0.1 to 0.7 g	5.0 mL
0.05 g	2.5 mL
0.029 g	1.5 mL
0.01 to 0.016 g	0.6 mL

2×10^8 cells	5.0 mL
5×10^7 cells	2.5 mL
2×10^7 cells	1.0 mL

Add the appropriate volume of lysis buffer to the tissue or cells. Grind, mince, or vortex, as is appropriate to the tissue. RNase and Proteinase K in the presence of SDS are typically added to remove RNA and protein. The lysate is incubated at 37 °C to allow action of these enzymes.[8,11,12]

10.3.7 Extraction of DNA

There are a number of different protocols for DNA extraction.[11,12] If properly performed, they should all yield high-quality DNA that is appropriate for gene rearrangement studies.

There are significant safety hazards that are associated with certain DNA extraction protocols, particularly those involving phenol/chloroform extraction methods. *Phenol causes severe burns*. Gloves, laboratory coats, and goggles are necessary for protection, as is the use of a certified fume hood. Upon contact, irrigate the affected area with water for 20 to 30 minutes. *Phenol vapors are toxic*. If a large spill occurs, evacuate the area and notify chemical hazard personnel. *Hydroxyquinoline is a possible carcinogen*. Handle with gloves and avoid contact with skin. Upon contact, irrigate the affected area with water for 20 to 30 minutes. Use of nonorganic extraction methods (some of which are commercially available as kits) can reduce these hazards.

10.3.8 Dialysis

When incomplete digestion of DNA is encountered, it can be useful to carry out dialysis after extraction, although it is not routinely performed. [11,12]

10.3.9 Precipitation of DNA

DNA is frequently precipitated to remove salts and decrease volume. Resuspension can be difficult, and it can affect yields. Avoid excessive drying of pellets, because this makes resuspension difficult. [11,12]

10.3.10 Resuspension

The volume of solvent required for resuspension depends on the amount of DNA in the pellet.

10.4 Assessing the Quantity and Quality of Extracted DNA

Based on absorbance, DNA can be quantitated using a properly calibrated spectrophotometer. Spectrophotometric quantification controls should be used per the manufacturer's instructions. To determine the quantity of DNA extracted, determine the A_{260} and A_{280} values on a dilution of the stock of resuspended DNA. The dilution should be in the linear range of the spectrophotometer, and the sample should be assayed in a clean, dry quartz cuvette. From the A_{260} readings of the dilution, one can calculate the DNA concentration of the stock solution and hence the microgram yield of DNA. This is based on 50 µg of double-stranded DNA per absorbance unit. From the A_{260}/A_{280} reading, one can determine the relative purity of the DNA solution with respect to protein contamination. Nucleic acids absorb maximally at A_{260}, while proteins absorb maximally at A_{280}. An A_{260}/A_{280} ratio of 1.8 to 2.0 indicates a high purity of DNA solution. If the ratio is below 1.6, reextraction and precipitation can increase the purity.[8]

From solid tissue, the yield will vary with the cellularity of the tissue used. From cellular tissue such as lymph nodes involved with non-Hodgkin's lymphoma, one can obtain 250 to 700 µg of DNA from 0.1 to 0.2 g of tissue, respectively. From human skin and human bowel, which are less cellular and contain more connective tissue, one may expect to obtain 30 to 1,000 µg of DNA from 0.03 to 0.72 g of tissue, respectively, and have more trouble with DNA degradation.

From cell pellets, the yields vary somewhat, which is presumably due to the viability of the cells at the time of DNA isolation. Assuming human cells contain 6×10^{-12} g (6 pg) of DNA per cell, one should obtain 30 to 90% of the theoretical yield. So, from 1×10^8 cells, obtain 200 to 550 µg of DNA; from 4×10^7 cells, obtain 100 to 200 µg of DNA; and from 2×10^7 cells, obtain 40 to 80 µg of DNA.

Using an agarose mini-gel, a visual comparison of the undigested sample DNA and a standard of known DNA concentration (such as a solution of lambda bacteriophage) can be made, if desired. Comparison of the results of both types of quantitation can occasionally yield discrepancies due to incomplete dissolution of genomic DNA in concentrated solutions. To ensure an accurate southern blot, a visual examination of a test gel allows for adjustment of the amount of DNA added to the restriction digests.[8]

Using this mini-gel, assessment of the integrity of the genomic DNA preparation can be made. Uncut, high-molecular-weight DNA should migrate only slightly into the gel, and it will remain as a tight band with minimal smearing. The presence of a "smear" extending from near the point of origin into the lower portion of the gel is indicative of degradation of the sample DNA. Samples showing significant degradation cannot be reliably analyzed. A photograph of the gel should be kept in the laboratory records or a notebook. [8]

10.5 Storage of DNA

DNA can be stored for one year (or perhaps longer) in tightly sealed containers at 2 to 6 °C. Extracted DNA can be stored as a frozen stock within tightly closed containers for periods exceeding seven years at -20 °C.

10.6 Digesting Genomic DNA with Restriction Endonucleases

To detect and identify the specific gene rearrangement indicative of a monoclonal population of a specific cell lineage, appropriate DNA probes must be hybridized to membranes containing the patient's DNA, which has been digested with a minimum of three appropriate restriction enzymes.

To achieve sufficient sensitivity in the detection of gene rearrangements, multiple restriction endonucleases are needed. To minimize the risk of missing a rearrangement that has generated a restriction fragment identical in size to the germline band seen with any single enzyme, genomic DNA samples should be digested with a minimum of three restriction endonuclease enzymes before electrophoresis. The most commonly used are *Eco*RI, *Bam*HI, *Hin*dIII, and *Bgl*II. There is no perfect or universally accepted enzyme combination. The laboratorian should use enzymes for which the laboratorian has a good understanding of the digestion characteristics as applied to this assay. A minimum of three different restriction enzymes is recommended. Maintain permanent records of samples, controls, marker lanes, and enzymes used for each gel run.[2,8]

The following combinations of probes and restriction digests are commonly used for T-cell receptor gene rearrangements and for B-cell immunoglobulin gene rearrangements:

- Immunoglobulin heavy chain (J_H and others): *Eco*RI, *Bam*HI, *Hin*dIII;
- Immunoglobulin light chain (J_λ, J_K, and others): *Eco*RI, *Bam*HI, *Hin*dIII; and
- T-cell receptor beta chain (C_β, J_β, and others): *Eco*RI, *Bam*HI, *Hin*dIII.

Restriction enzymes are stable when they are stored at -16 to -24 °C in buffer containing 50% glycerol. To preserve enzyme integrity, avoid extended periods of exposure to room temperature (22 to 25 °C).

The restriction enzyme volume must contribute less than 10% of the final reaction volume, or the remaining glycerol can interfere with the assay.

10.6.1 Setting Up Digestions with Restriction Enzymes

Genomic DNA reactions, used for detecting single-copy sequences, typically contain 5.0 to 10.0 µg of DNA, spermidine (1 mmol/L), and restriction enzyme (in a final reaction volume of 20 to 200 µL).[8]

10.6.2 Addition of Restriction Enzyme

To ensure that it is in the proper buffer, add the restriction enzyme last. The precise enzyme quantities necessary should be validated under standard conditions in the individual laboratory. Enzyme activities used typically include:

- *Eco*RI digests: 20 units/5 µg genomic DNA;
- *Bam*HI digests: 75 units/5 µg genomic DNA;
- *Hin*dIII digests: 40 units/5 µg genomic DNA; and
- *Bgl*II digests: 50 units/5 µg genomic DNA.

10.6.3 Incubation of DNA with Restriction Enzyme

Although three hours is a common incubation time, longer incubation (up to overnight) typically does not degrade test performance. Digestion with excess enzyme does not cause problems, unless there is contamination with DNase or exonuclease. The restriction enzyme volume must contribute less than 10% of the final reaction volume, or the remaining glycerol can interfere with the assay by inducing "star" activity (i.e., loss of sequence specificity) in the restriction enzyme.

10.6.4 Stopping Restriction Digests

Various methods can be used to stop restriction enzyme digests. Two methods include:

(1) adding concentrated loading dyes and immediately loading the agarose gel; and

(2) stopping the reaction by freezing and storing at -16 to -24 °C, or by adding 0.5 mol/L EDTA (pH 7.5) to a final concentration of 10 mmol/L.

10.7 Preparation of Southern Blots

To detect changes in gene organization, the restricted DNA strands must be gel-fractionated to resolve the DNA according to its molecular weight.

The DNA is then transferred onto a nitrocellulose or nylon membrane, which can later be hybridized to the appropriately labeled probe.[8,14]

10.7.1 Gel Electrophoresis

Electrophoresis is used to separate digested DNA according to size. Optimally, restriction enzyme digests will be carried out in sufficiently small volumes to allow direct loading. However, the volume of the digest can be reduced by ethanol precipitation and resuspension in an appropriate volume of Tris/EDTA (TE) or running buffer. Because the DNA will not be directly quantitated after this step, potential losses can go undocumented with precipitation.

Typically, 0.7 to 1% agarose is used to achieve separation in the size range of 2 to 20 kilobases (kb).[8]

10.7.2 Controls

Each gel should contain one or two lanes (depending on the width of the gel and the number of samples loaded) of a molecular size marker with fragments spanning 2 to 20 kb. The marker can be end-labeled with a radioactive nucleotide to permit direct visualization of the bands on an autoradiogram.[8]

Negative control DNA samples may be prepared from any sample lacking rearrangement or polymorphism at the locus of interest, such as placental DNA, DNA from a cell line, or a known normal sample. To provide a control for the digestion, negative controls should be digested with the same restriction endonuclease enzymes as patient samples. The negative controls will provide a demonstration of the germline band sizes for each restriction endonuclease.[8]

Positive controls should provide an example of rearrangement for each of the restriction enzymes used, and they should also permit assessment of the sensitivity of each hybridization and resultant autoradiogram. Positive controls may consist of DNA from a known positive sample or a cultured malignant cell line diluted or mixed proportionally with germline-negative control DNA to provide a sensitivity control of 10% on each gel. Additional sensitivity controls at greater dilutions may also prove useful.

An alternative approach to the preparation of a sensitivity and an unrearranged negative control is to digest two aliquots of DNA separately with two different restriction endonucleases, then mix one into the other at a ratio of 1:10. When loaded onto a gel, a single lane of DNA can thus serve as the negative control for each of two restriction digests, as well as the sensitivity control.

10.7.3 Running the Gel

Voltage should be low (e.g., 1 to 2 V/cm) and runs should be long (18 to 24 hours). Longer runs at lower voltage give better fragment separation. For example, with 0.7% agarose gel:

(1) For T_β with *Bam*HI, and J_H with *Eco*RI, 22 to 24 hours at ~2 V/cm (35V) places a 3-kb band at ~10 cm of migration and can be optimum.

(2) For Tγ with *Bam*HI and *Eco*RI, and T_β with *Eco*RI and *Hin*dIII, 22 to 24 hours at ~1.25 V/cm (22V) places a 0.5-kb band at 10 cm of migration and can be optimum.

Recirculation of buffer can improve results.

10.7.4 Visualization of DNA

DNA stained with ethidium bromide fluoresces under ultraviolet (UV) light, which allows visualization and photography of DNA size markers and digested specimen DNA.

The DNA in each lane should appear equivalent in amount, and it should be seen as an even smear indicative of complete digestion. Following southern blot transfer, the gel may be restained with ethidium bromide, illuminated with UV light, and photographed to document complete transfer of DNA for laboratory records. A photograph is not mandatory; the technologist may simply document that the completeness of transfer was verified by visualization with UV light.

10.7.5 Transfer of DNA to Rigid Support (Blotting)

After DNA has been fractionated by gel electrophoresis based on size, it is immobilized in this configuration and affixed to a solid support for hybridization with probes by transfer to a nylon or nitrocellulose membrane.

The steps outlined below are typically employed.

10.7.5.1 Depurination

To improve transfer, DNA is depurinated by exposure to acid. High-molecular-weight DNA can be depurinated by exposure to a depurination solution, followed by distilled water. Insufficient depurination leads to inefficient transfer; overdepurination can damage the DNA. The time required for depurination depends on the gel concentration and thickness, and it typically ranges from 15 to 30 minutes. Each laboratory should experimentally verify the proper depurination times for each type of gel used in that laboratory. [8]

10.7.5.2 Denaturation

The gel is denatured, typically by soaking in NaOH for 30 minutes at room temperature (22 to 25 °C) with gentle to moderate agitation. This step may be repeated to ensure complete denaturation. The DNA can then be transferred directly to a nylon membrane using NaOH as a transfer buffer (alkaline transfer). An NaOH concentration of 0.4 to 0.5 mol/L is most often used for alkaline transfer. [8]

10.7.5.3 Neutralization

If a nonalkaline transfer is used, the gel must be neutralized before transfer. This is accomplished by washing the gel repeatedly with a Tris buffer for 30-minute intervals. [8]

10.7.5.4 Transfer

Digested and size-fractionated DNA is transferred from the gel to nylon membrane or nitrocellulose. The DNA is carried to the membrane by mass movement of buffer across the gel and through the membrane by vacuum or capillary action. Air bubbles or other barriers to transfer cause failure of the transfer.

Vacuum transfer requires less time (approximately three hours), but it requires equipment that is specific to this purpose.

Capillary-action transfer requires approximately 18 hours or longer. No specific equipment is necessary. Briefly, the gel is suspended above a reservoir containing a buffer [e.g., 2x or 10x standard sodium citrate (SSC)] or NaOH, with wicks of filter paper that carry the fluid beneath the gel. The membrane is placed on top of the gel, and dry filter paper is placed on top of the membrane. The dry filter paper on top pulls the buffer up through the gel and membrane by capillary action. To maintain capillary action, dry filters must remain on top until the conclusion of transfer. [8]

10.7.5.5 Check of Transfer

Completeness of the transfer of DNA can be checked by using UV light to illuminate the ethidium bromide-stained gel and checking for any residual DNA. With alkaline transfer, gels must first be neutralized. Because these factors will affect interpretation of the data, bubbles or incomplete transfer should be documented.

10.7.5.6 Wash and Binding to Membrane

Following transfer, the membrane is washed to remove adherent agarose and, in the case of alkaline transfer, to neutralize the pH. The membrane, or blot, is dried or UV cross-linked to permanently affix the DNA to the membrane. Nitrocellulose blots may be air-dried for 8 to 12 hours, or baked under vacuum for at least two hours at 75 to 85°C. Nylon membranes are cross-linked under ultraviolet light. Once dried or cross-linked, blots can be stored indefinitely at room temperature (22 to 25 °C) or at 2 to 6 °C. [8]

The membrane should be labeled for future reference and identification of diagnostic results on specimens.

10.8 Probes

Probes can be obtained commercially or from other laboratories.

10.8.1 Radiolabeling Probes

Probes are labeled to allow the DNA bands hybridizing to the probes to be visualized. Either radiolabeled probes or probes labeled with nonisotopic markers can be used in gene rearrangement assays.

A radiolabeled probe provides the sensitivity needed to detect one gene [approximately 1,000 base pairs (bp) out of the human genome, which is 3×10^9 bp] or to detect better than one out of a million sequences. Radioactive material must be handled according to Nuclear Regulatory Commission regulations, which include strict safety requirements. All radiolabeling procedures use enzymes to

incorporate radioactive nucleotides into probe sequences. Typical protocols include nick translation, random priming, and end-labeling. [8]

10.8.2 Purification of Labeled Probes

Purification is used to remove free radioactive nucleotides that create excessive background upon hybridization. Purification employing separation columns is commonly used; smaller radioactive nucleotides are retained in the pores and the larger, excluded, labeled probes are eluted.

10.8.3 Quantitation of Incorporation/Final Specific Activity Determination

Trichloroacetic acid (TCA) precipitates the larger probe; counting TCA-precipitated material in a scintillation counter allows quantitation of the labeling of probe DNA. Typically, samples to be precipitated are spotted onto glass fiber filters, washed repeatedly with 10% TCA at 4 EC, air dried, and counted.

Because the input mass of DNA is known for the reaction, precolumn quantitation numbers are best used to determine the specific activity of the probe. An aliquot is removed, precipitated using TCA, and filtered. The clumped, precipitated material is retained on the filter, which is counted for determination of radioactivity.

Postcolumn quantitation numbers indicate the amount of incorporated count available as radioactive DNA probes and indicate whether the column has succeeded in removing unincorporated radioactive nucleotides.

10.8.3.1 Calculation of Specific Activity

Calculate the total precolumn precipitable cpm (e.g., if a 5-µL aliquot of 100 µL total volume is precipitated by TCA, multiply the counts in that 5-µL aliquot by 20), and then divide by the input number of micrograms of DNA. The specific activity should be approximately 1×10^8 to 8×10^8 cpm/µg.

10.8.3.2 Calculation of Percent Incorporation

Divide the precolumn precipitable count by the precolumn total count. The percent precipitable generally ranges from 25 to 75%.

10.8.3.3 Calculation of Postcolumn Purity

Divide the postcolumn precipitable count by the postcolumn total count. If the column has removed most of the unincorporated nucleotide, the postcolumn total and precipitable counts will be nearly equal. Thus, the postcolumn percent precipitable will be approximately 85 to 95%.

10.8.4 Nonradioactive Labeling

Although nonradioactive labeling of probes can be done in the laboratory, most laboratories that use nonradiolabeled probes obtain them from an FDA-approved test system. These probes should be validated and used according to the manufacturer's instructions.

10.9 Filter Hybridization

Labeled DNA probes that contain sequence homology to DNA that is of diagnostic interest are hybridized under conditions that promote annealing of the probes to complementary regions of restriction-digested DNA. The procedure consists of prehybridization, hybridization, washing, and visualization steps.

10.9.1 Prehybridization

Prehybridization prepares the membrane for hybridization with a labeled probe and decreases the nonspecific binding of a probe by preblocking nonspecific sites with short fragments of nonmammalian DNA. Prehybridization is performed for 2 to 12 hours at the same temperature (and usually with the same buffer) as hybridization. [8]

10.9.2 Hybridization

Hybridization is continued for 24 to 36 hours at a temperature that promotes annealing of the probe, without significant nonspecific binding. In the presence of a denaturing agent, such as formamide, this temperature is typically 42 °C for genomic probes or lower for oligonucleotide probes. If denaturing agents are not used, the appropriate temperature for hybridization may be 65 to 70 °C or higher. Agitation, such as that provided by a rocking platform, provides constant mixing. To produce single strands of DNA, the probe is denatured before being added to the hybridization buffer. When hybridization is complete, the probe can be saved for immediate reuse (within a few days), or can be discarded in radioactive waste. [8]

10.9.2.1 Probe Denaturation

Denaturation is accomplished using heat. A sample procedure involves heating the solution containing the probe to 100 °C for three to five minutes, then immediately diluting the solution containing denatured probe with 2 to 3 mL of preheated (approximately 42 to 50 °C) hybridization buffer. Alternatively, the probe may be denatured using NaOH that has been diluted to prevent strand reassociation; the solution can then be neutralized, usually with acid followed by Tris buffer.

10.9.2.2 Bubbles

Bubbles should be avoided; they can interfere with probe hybridization.

10.9.2.3 Volume of Hybridization Buffer

The volume of hybridization buffer used depends on the size of the membrane and the container used for hybridization. For effective wetting of the membrane and for adequate mixing of buffer throughout the hybridization process, larger membranes require larger volumes of hybridization buffer. Because they have a larger surface area that must be kept wet (and for effective mixing of the buffer), heat-sealed plastic bags require a larger volume (20 to 40 mL) than cylindrical tubes for hybridization ovens (8 to 20 mL).

10.9.2.4 Quantity of Labeled Probe

The quantity of labeled probe necessary is affected by a number of considerations, including the nature of the probe; the volume of hybridization media; the method of labeling; the specific activity of the probe; and the size of the blot. As a result, individual laboratories must carefully validate the quantity of labeled probe.

10.9.3 Washing

The membranes are washed several times to remove nonspecifically bound probe. The first wash, to remove adherent hybridization buffer, is typically a low-stringent wash of short duration, such as 15 minutes. To remove any labeled probe not annealed to the specific target DNA sequence, one or several stringent washes with low salt and higher temperatures are then performed for 30 minutes to 1 hour. The

desired temperature and salt concentration depends on the melting point of the probe. Running a Geiger counter over the blot is helpful in determining if the blot was washed sufficiently; normally, less than a few hundred counts should be detectable on the surface of the radioactive filter.

10.9.4 Visualization of Bands

To visualize radioactive-labeled probes, the washed membrane should be blotted dry (excess fluid may quench the radioactive signal), wrapped in a light, waterproof cover (e.g., plastic wrap) to prevent radioactive contamination, and placed in a film cassette with x-ray film at -70 ± 10 °C for two days to two weeks. Intensifying screens in the film cassette can increase the sensitivity of detection by increasing the exposure of the film. Exposing the screen to radioactive emissions at -70 ± 10 °C results in light production. High backgrounds indicate the need for further washing, perhaps at a higher stringency.

The autoradiographic films should be maintained permanently in the laboratory files or notebook. The film should be labeled with the date, probe used, and sample and control identification. The film should be free of background and extraneous spots, which will obscure the sample results. Germline and rearranged bands should be clearly visible in each lane with a minimum of "smiling" or smearing. The 10% sensitivity controls should be visible. Repeat autoradiography if the film is unsatisfactory.

Many laboratories are moving away from x-ray film toward digital records obtained from equipment such as a phosphoimager. Use of software to modify apparent intensity and background (allowing faint bands to be evaluated fully) should be documented in the laboratories' records. Retention of hard copies of the digital data, in the form that the data are interpreted, is strongly recommended. Because of the rapid evolution of digital technology, many media that were recently available can no longer be read on current computers; similar evolution and migration can be expected in the future. We believe that at this time the retention of a faithful hard copy of what was interpreted at the time of "sign out" is a reasonable approach to deal with this problem.

11 Interpretation of Gene Rearrangement Studies

Southern blots must be free from excessive background hybridization signal. Southern blots must also permit recognition of a clonally rearranged allele contributed by a cell population in which fewer than one in ten cells are clonally derived. In southern blots with some background hybridization, care must be taken to ensure that an apparent allele actually falls within the boundaries of a sample lane run on the gel.

11.1 Acceptable Evidence for a Clonally Rearranged Allele

The presence of a distinct, even if faint, band at a migration distance that is different from that of germline alleles or known "regular" or "irregular" bands (see following) is considered presumptive evidence for a clonally rearranged allele.[2]

For a definitive diagnosis of rearrangement to be made, results must be internally consistent. Lanes from restriction digests based on at least two different restriction endonucleases should show evidence of clonal rearrangement. In some cases, true gene rearrangement may be manifested as two rearranged bands demonstrated by a single enzyme digest, while other restriction digests show only germline patterns. However, when only a single restriction digest demonstrates clonal bands, great care must be exercised when interpreting a gene rearrangement assay as demonstrating clonal rearrangement.[2] An additional assay, based upon the use of a fourth restriction endonuclease, such as *Bg*/II, may be run for verification when only a single rearranged band is observed.

11.2 Characterization of Rearrangements

To facilitate reporting of laboratory results, interlaboratory comparisons, and comparisons of results obtained on different specimens from a single patient, determination of sizes and estimation of intensities of rearranged alleles is recommended.

11.2.1 Size Determination

The size of rearranged alleles should be determined from semilogarithmic plots or estimated by comparison with the migration of standards of known molecular size. Sizes of rearranged alleles should be reported.

11.2.2 Estimating the Size of a Clonal Population

From hybridization intensities, it may be possible to roughly (±20%) estimate the percentage of clonal cells in the population analyzed. Densitometry with comparison to known controls is helpful in making these estimates of the percentage of clonal cells.

11.3 Interpretation of Cross-Hybridization Bands

The laboratory should have written guidelines for the interpretation of faint bands that regularly appear in regions where cross-hybridization with presumably nonimmunoglobulin or T-cell receptor genes is routinely known to occur. These include: [8]

Ig J_H probe:

- a band at ~20 to 22 kb with *Eco*RI;
- a band at ~3.5 kb with *Hin*dIII (sometimes polymorphic); and
- a band at ~10 kb with *Bam*HI.

$TCR_\beta 3$ probe:

- a band at ~9 kb with *Eco*RI (partial); and
- a heterodisperse smear in the region around 12 to 14 kb with *Bam*HI.

11.4 Interpretation of Anomalous Bands

The laboratory should have written guidelines for the interpretation of anomalous bands or artifacts that occur irregularly without consistent association with either benign or neoplastic processes. These include the following:

- Rare restriction fragment-length polymorphisms with Ig J_H using the enzymes *Hin*dIII (10 and 9 kb) or *Bgl*II. Bands at ~6 and ~3 kb with *Eco*RI probed with TCR_β (rare).
- Upward-deflected "wings" on hybridizing genomic bands, particularly in overloaded lanes. If prominent and jointed, these may simulate a faintly hybridizing allele above a more prominently hybridizing band.

- In lanes on southern blots that contain modestly underloaded samples, germline bands can appear to migrate somewhat more slowly than normal.

- Contamination of probe and/or samples, illustrated by an unexpected, usually quite darkly hybridizing allele in multiple (usually all) samples.

11.5 Interpretation of Clonal Rearrangement (Test Limitations)

Laboratories must develop procedures for assessing the presence of clonal rearrangements in terms of the biology of the diseases giving rise to these rearrangements. In particular, laboratory diagnosis of disease based on the presence or absence of monoclonal immunoglobulin or T-cell receptor gene rearrangements must consider the following:

- A malignant neoplasm characterized by monoclonal gene rearrangement will not be detected if an insufficient number of malignant cells are present in the sample.

- The presence of a monoclonal gene rearrangement does not necessarily reflect the presence of a lymphoid neoplasm. Transient clonal proliferation can occur, particularly in immunocompromised patients.

- Incomplete or partial digests can yield a southern blot that simulates that of a biopsy that demonstrates a monoclonal rearrangement.

- Certain genes, particularly T_c and T_d, have few possible rearrangements. Blots based on these genes frequently appear to demonstrate a monoclonal band when the lymphoid population present is, in fact, polyclonal.[2]

- Occasionally, B-cell neoplasms demonstrate rearranged T-cell receptor genes, while, occasionally, T-cell neoplasms demonstrate rearranged immunoglobulin genes. On occasion, myeloid (and perhaps other) neoplasms can also demonstrate immunoglobulin or T-cell receptor gene rearrangement. Often, this phenomenon is termed "lineage infidelity."[2,16,17,18,19]

12 Reporting of Gene Rearrangement Studies

Reports should contain the following:

- patient identification information, including last name, entire first name, and middle initial. (Since family members often share these names, it is ideal if the date of birth is also included on the report, as well as any unique patient identification numbers.);

- laboratory identification number;

- name of the referring physician;

- date of specimen collection and receipt;

- nature of sample (peripheral blood, bone marrow, lymph node, paraffin block, etc.);

- date of the report, primary delivery address for the report, and an indication of to whom other copies of the report have been sent;

- identification of the laboratory, including appropriate state and other license numbers, and a telephone number to call for professional consultation about study interpretation and implications for patient care;

- results, including a description of the test(s) performed, probes employed, restriction enzymes used, and a clear description of any abnormal results or significant features that affect interpretation of the study, such as limited sample DNA, degraded sample DNA, or incomplete digestion;

- an interpretation of the results (Ideally, this is done as part of a staged multiparameter work-up in which histologic, immunologic, and possibly cytogenetic and/or cytochemical information is also available.); a statement addressing test sensitivity should be included; and

- signature of the reporting physician or scientist.

Other information that is valuable and can be included on reports includes a clinical synopsis and reason for study; reference to supporting laboratory information (such as results from histologic, immunologic, and cytogenetic studies, if these are not included as part of the result interpretation); the referring clinician's phone number; and a photograph or computer image of the actual southern blot results.

References

[1] Cossman J, Uppenkamp M, Sundeen J, et al. Molecular genetics and the diagnosis of lymphoma. *Arch Pathol Lab Med.* 1988;112:117-127.

[2] Cossman J, Zehnbauer B, Garrett CT, et al. Gene rearrangements in the diagnosis of lymphoma/leukemia. Guidelines for use based on a multiinstitutional study. *Am J Clin Pathol.* 1991;95:347-354.

[3] Grody WW, Hilborne LH. Diagnostic applications of recombinant nucleic acid technology: neoplastic disease. *Lab Med.* 1992;23:19-23.

[4] Hanson CA, Ross CW. The molecular genetics of the immunoglobulin and T-cell receptors: applications in diagnostic hematopathology. In: Fenoglio-Preiser C, et al, eds. *Advances in Pathology, Vol. 3*. St. Louis: Mosby Year Book. 1990:33-74.

[5] Knowles DM, Pellici PG, Dalla-Favera R. Immunoglobulin and T-cell receptor beta chain DNA probes in the diagnosis and classification of human lymphoid neoplasia. *Mol Cell Probes.* 1987;1:15-31.

[6] Madeiros LJ, Bagg A, Cossman J. Application of molecular genetics to the diagnosis of hematopoietic neoplasms. In: Knowles DM, ed. *Neoplastic Hematopathology*. Baltimore: Williams and Wilkins. 1992:269-271.

[7] Whitaker MH, Willman CL. A practical guide to the use of molecular genetic techniques in the diagnosis of lymphoid malignancies. In: Fenoglio-Preiser CM, Willman CL, eds. *Molecular Diagnosis in Pathology*. Baltimore: Williams and Wilkins. 1991:123-148.

[8] Farkas DH. Specimen procurement, processing, tracking and testing by the southern blot; Quality control of the B/T cell gene rearrangement test; and Procedure manual for the B/T cell gene rearrangement test and bcr analysis. In: Farkas DH, ed. *Molecular Biology and Pathology, A Guidebook for Quality Control*. San Diego: Academic Press. 1993:51-75;77-101;233-286.

[9] Korsmeyer SJ, Arnold A, Bakhshi A, et al. Immunoglobulin gene rearrangement and cell surface antigen expression in acute lymphocytic leukemias of T cell and B cell precursor origins. *J Clin Invest.* 1983;71:301-313.

[10] Todd DM, Buccini FJ. Apparent heparin interference with restriction enzyme digestion of genomic DNA. *Clin Chem.* 1993;39:362-363.

[11] Davis LG, Dibner MD, Battey JF. *Basic Methods in Molecular Biology*. New York: Elsevier; 1986.

[12] Maniatis T, Fritsch EF, Sambrook J. Purification of nucleic acids. In *Molecular Cloning, A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory. 1982:458-462.

[13] Southern EM. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol.* 1975;98:503-517.

[14] Feinberg AP, Vogelstein B. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem.* 1983;132:6-13.

[15] Harrington DS. Molecular gene rearrangement analysis in hematopathology. *Am J Clin Pathol.* 1990;93(suppl 1):S38-S43.

[16] Cossman J, Madeiros LJ. Lymphoproliferative diseases. In: Cossman J, ed. *Molecular Genetics in Cancer Diagnosis*. New York: Elsevier. 1990:189-202.

[17] Weiss LM, Sklar JL. T cell neoplasms. In: Cossman J, ed. *Molecular Genetics in Cancer Diagnosis*. New York: Elsevier. 1990:203-222.

[18] Kitchingman GR, Mirrow J, Stass SA. The molecular biology of mixed-lineage leukemia. In: Cossman J, ed. *Molecular Genetics in Cancer Diagnosis*. New York: Elsevier. 1990:223-248.

[19] Hanson CA, Thamilarasan M, Ross CW, et al. Kappa light chain gene rearrangement in T-cell acute lymphoblastic leukemia. *Am J Clin Pathol.* 1990;93:563-568.

Appendix. Probes

A1. J probe – pHuJ$_H$ [1]

2.7 kb Sau 3A fragment derived from the 6 kb *Bam*HI-*Hin*dIII genomic insert into pBR322 (contains genomic DNA J2 through J6) [germline *Eco*RI = 16 kb single band]

A2. J$_k$ probe – pHuJ$_k$ [2, 3]

1.8 kb Sac I fragment from a 1.8 kb Sac I genomic insert into a Sac I vector (contains 5 J_K exons and intervening sequences) [germline *Bam*HI digest = 12 kb only]

A3. Cλ probe – HuCλ [4]

0.8 kb *Eco*RI fragment from a 0.8 kb *Eco*RI genomic insert into pBR322 vector (contains Cλ) [germline *Eco*RI = 16 kb, 14 kb, 5 kb pseudogene, and polymorphic 8 kb (which can be replaced by a 13-kb, 18-kb, or 23-kb band)]

A4. T$_\beta$ probe - HuTCR$_\beta$ [5]

0.75 kb Pst I-AvaI fragment derived from a 770 bp cDNA insert by GC tailing into pBR322 (contains J, mostly C sequences) [*Eco*RI germline = 4.2 & 12 kb; *Bam*HI germline - 24 kb with C region probe; *Hin*dIII germline - 7.2 to 8; 6.5 to 4; 3.5 to 2.5]

A5. T$_\gamma$ probe - pT$_\gamma$l [6]

1.4 kb *Eco*RI fragment derived from a 1.4 kb cDNA insert into pUC9 (contains V, J, and C regions) [germline *Eco*RI = 9.5 kb, 7.5 kb, 6.7 kb, ~5-6 kb, 3.8 kb, 3.1 kb, 2.5 kb, 1.9 kb; *Bam*HI germline = ~24 kb, ~15 kb, ~4 kb]

A6. T$_\alpha$ probe - HuTCRα - pGA5 [7]

1.3 kb *Eco*RI fragment derived from a 1.3 kb cDNA insert into pUC13 (contains 5' unt, V, J, C, 3' unt sequences) [germline *Bam*HI = ~6.6 kb, ~5 kb, and f~3.5 kb; PVUII; germline = ~4.5, ~5 kb, and ~<l kb]

A7. T$_z$ = HuTC$_z$VJC = 0-240/38 [8]

0.43 kb *Eco*RI fragment cDNA insert into pUC18 (contains V, J, and a little C sequence) [*Eco*RI germline bands = ~1.5 kb, ~3.3 kb, ~6.6 kb; PVUII germline bands = 2.0 kb, 6.6 kb, 23.0 kb]

References

1. Ravetch JV, Siebenlist U, Korsmeyer S, Waldmann T, Leder P. Structure of the human immunoglobulin mu locus: characterizations of Embryonic and rearranged J and D genes. *Cell*. 1981;27:583-591.

2. Heiter PA, Max EE, Seidman JG, Maizel JV Jr., Leder P. Cloned human and mouse kappa immunoglobulin constant and J region genes conserve homology in functional segments. *Cell*. 1980;22:197-207.

3. Heiter PA, Maizel JV Jr., Leder P. Evolution of human immunoglobulin kappa J region genes. *J Biol Chem*. 1982;257:1516-1522.

4. Heiter PA, Hollis GF, Korsmeyer SJ, Waldman TA, Leder P. Clustered arrangement of immunoglobulin lambda constant region genes in man. *Nature*. 1981;294:536-540.

5. Yoshikai Y, Anatoniou D, Clark SP, et al. Sequence and expression of transcripts of the human t-cell receptor beta-chain genes. *Nature*. 1984;312:521-524.

6. Quertermous T, Murre C, Dialynas D, et al. Human t-cell gamma chain genes: organization, diversity, and rearrangement. *Science*. 1986;231:252-255.

7. Sim GK, Yague J, Nelson J, et al. Primary structure of human t-cell receptor alpha-chain. *Nature*. 1984;312:771-775.

8. Hata S, Brenner MB, Krangel MS. Identification of putative human t-cell receptor delta complementary DNA clones. *Science*. 1987;238:678-682.

NCCLS consensus procedures include an appeals process that is described in detail in Section 9 of the Administrative Procedures. For further information contact the Executive Offices or visit our website at www.nccls.org.

Summary of Comments and Subcommittee Responses

MM2-A: *Immunoglobulin and T-Cell Receptor Gene Rearrangement Assays; Approved Guideline*

General

1. Wording of some definitions, i.e., DNA, nucleic acid – but I realize these were drawn up by "committee."

- **For consistency, the definitions used in MM2 have been aligned with NCCLS document NRSCL8—*Terminology and Definitions for Use in NCCLS Documents*. NRSCL8 was not available at the time of publication of MM2-A.**

2. There is no statement to the effect that an insert from a manufacturer's kit is NOT adequate for a procedure.

- **This is found elsewhere in NCCLS documents, and does not seem to need restating. The recommendations provided herein do not preclude the use of a "package insert" for a procedure.**

3. There is no indication of who is qualified to "sign-out," though in Section 12 (formerly Section 11) it is stated that the report should have the "signature of the reporting physician." In many cases, these studies are being performed and interpreted by doctoral level scientists. Are they eligible/ineligible to "sign-out"?

- **Occupational qualifications are likely to vary from country to country. For clarification, the wording has been modified to read "reporting physician or scientist."**

Section 10.3.5 (formerly Section 9.3.5)

4. In the first paragraph, 20 to 40 mL of whole blood is recommended for DNA extraction. We routinely use only 3 mL of peripheral blood for the extraction and recommend about 10 mL be collected. For bone marrow, we require only 1 to 2 mL, not up to 5 mL as recommended in the guidelines.

- **The committee agrees with the recommendation. The text has been modified as suggested.**

Section 10.3.5.1 (formerly Section 9.3.5.1)

5. The recommendation to centrifuge at 100 rpm: Is this with any rotor radius? Preferably give rcf. I would suggest that the text be reworded as follows: "Bring the PBS up to 50 mL and centrifuge at low speed (typically around 100 rpm)." The reason for this is that it really doesn't make all that much difference. We want to sediment the cells without packing them so tightly they cannot be separated for further washing.

- **The committee agrees with the recommendation. The text has been modified as suggested.**

Section 10.6 (formerly Section 9.6)

6. This section recommends the use of particular restriction enzymes. Despite the widespread use of the recommended enzymes, other enzyme combinations actually may be better choices for diagnostic purposes. For instance, the hybridization of EcoR1 and HindIII digests with probes to the immunoglobulin heavy chain has been discouraged on the grounds that the presence of VNTRs within the hybridizing fragments may result in restriction fragment length polymorphisms. (Beishuizen A, et al. Detection of immunoglobulin heavy-chain gene rearrangements by southern blot analysis: Recommendations for optimal results. *Leukemia*. 1993;7(12):2045-2053.) The report also illustrates the advantage of choosing enzymes that result in smaller hybridization fragments.

- **In response to the commenter's concern, the following sentence has been deleted:**

  "Because these are the most widely used and there is the most experience with them, as a minimum, the three enzymes, EcoR1, BamH1, and HindIII are recommended."

  It has been replaced with the following:

  "There is no perfect or universally accepted enzyme combination. The laboratorian should use enzymes for which the laboratorian has a good understanding of the digestion characteristics as applied to this assay. A minimum of three different restriction enzymes is recommended."

Section 10.6.1 (formerly Section 9.6.1)

7. "(1mM)" Is this mmol/L?

- **The designation "1mM" is indicative of "millimolar," which is "mmol/L." The designation has been changed to "mmol/L" for clarity.**

Section 10.6.3 (formerly Section 9.6.3)

8. When stating that excess enzyme added to a reaction does not usually cause problems, the possibility of inducing star activity in the digest by excess glycerol should be pointed out, since this can affect the specificity of restriction enzyme activity.

- **In response to the commenter's concern, the last sentence of this section has been modified to read as follows:**

  "The restriction enzyme volume must contribute less than 10% of the final reaction volume, or the remaining glycerol can interfere with the assay by inducing "star" activity (i.e., loss of sequence specificity) in the restriction enzyme."

Section 10.7.3 (formerly Section 9.7.3)

9. Electrophoresis conditions should be expressed as field strength, in volts/centimeter under constant voltage conditions. A particular voltage will give different results in different apparatus, depending upon the distance between the electrodes in the apparatus.

- **As suggested, voltage designations have been expressed in V/cm.**

Section 10.7.4 (formerly Section 9.7.4)

10. Following restriction digestion of samples, we always run a small aliquot of the digest on a "test gel" to check visually for complete digestion. The gel is stained with ethidium bromide and photographed. If the DNA is not completely digested, then more enzyme is added and incubation continued. When a gene rearrangement is detected by southern analysis, this photograph is useful to verify that the DNA was digested completely. Sometimes the DNA appears to be digested after ethidium bromide staining but in fact contains some partial digestion fragments. In order to determine whether or not an abnormal restriction fragment pattern actually represents a gene rearrangement, we have become familiar with incomplete digestion patterns on a southern blot.

- **The committee appreciates the commenter's input. This statement is clearly one objective of doing an ethidium stain.**

Section 10.7.5.6 (formerly Section 9.7.5.6)

11. The section states, "…baking under vacuum…might not be necessary if nylon membranes are used…" This wording is vague; it should be changed to reflect the fact that standard practice for nylon membranes is to UV cross-link the DNA to the membrane.

- **The section has been reworded as follows:**

  "Following transfer, the membrane is washed to remove adherent agarose and, in the case of alkaline transfer, to neutralize the pH. The membrane, or blot, is dried or UV cross-linked to permanently affix the DNA to the membrane. Nitrocellulose blots may be air-dried for 8 to 12 hours, or baked under vacuum for at least two hours at 75 to 85 ^{o}C. Nylon membranes are cross-linked under ultraviolet light. Once dried or cross-linked, blots can be stored indefinitely at room temperature (22 to 25 ^{o}C) or at 2 to 6 ^{o}C."

Summary of Delegate Comments and Committee Responses

MM2-A2: *Immunoglobulin and T-Cell Receptor Gene Rearrangement Assays; Approved Guideline—Second Edition*

General

1. I question the retention of records in DNA for ten years. This is a good idea; however, it exceeds current JCAHO and CAP requirements.

- **The committee appreciates the commenter's concern and understands that the record retention recommendation is longer than that of some requirements. The recommendation was made as a compromise between the administrative desire to not retain records for an excessive period, and the clinical desire to have them available for comparison over the entire time frames during which lymphomas typically evolve.**

Section 6.2

2. I would also add to items on the requisition form B-cell or T-cell gene rearrangement test (or both) are being requested.

- **Although there is clearly no objection to using a requisition that lists all test types, there is a belief that for some laboratories this may yield an excessively long requisition form.**

Section 10.3.10

3. We have found that the method of resuspension as well as the buffer utilized influence the day-to-day A260 and A280 readings. Similar findings have been published (*Biotechniques*, 1997 Vol 22:474 and *Int J Mol Med*, 2000 5:657). These fluctuations impact the purity evaluation and the concentration determination. As these determinations are crucial to downstream step, procedures should be at least consistent within a laboratory. Guidance should be introduced at these steps to assure pellets are dissolved (suggestions on final concentration, buffer usage, warming specimens, etc.). Steps should be taken to assure buffers used for spectrophotometry are appropriate to allow consistent (day-to-day and lot-to-lot) evaluation of nucleic acid. These steps can prevent unnecessary extractions, and under- and overloaded gels, as well as provide consistency across lanes.

- **The committee agrees with the comments, but does not believe that this issue needs to be specifically addressed in more detail in this document. The objective of laboratory standard operating procedures (SOPs) is to assure consistency. A properly constructed and utilized set of SOPs should obviate the problems of intralaboratory inconsistency in work practice.**

Section 10.9.4

4. Many laboratories are moving away from x-ray film toward digital records obtained from equipment such as a phosphoimager. This method dramatically reduces the amount of time blots need to be exposed. Software allows for the modification of intensity and background allowing faint bands to be evaluated fully. As with digital media, manipulation of the data should be safeguarded. Is there a need to address this at this time?

- **Clearly this is important. The committee recommends retention of hard copies of the digital data, in the form that the data are interpreted. Because of the rapid evolution of digital technology, many media that were available ten years ago can no longer be read on current machines. At this time, the committee believes the retention of a faithful hard copy of what was**

interpreted at the time of "sign out" is a reasonable approach to addressing the issue. The following text has been added for clarification: "Many laboratories are moving away from x-ray film toward digital records obtained from equipment such as a phosphoimager. Use of software to modify apparent intensity and background (allowing faint bands to be evaluated fully) should be documented in the laboratories' records. Retention of hard copies of the digital data, in the form that the data are interpreted, is strongly recommended. Because of the rapid evolution of digital technology, many media that were recently available can no longer be read on current computers; similar evolution and migration can be expected in the future. We believe that at this time the retention of a faithful hard copy of what was interpreted at the time of 'sign out' is a reasonable approach to deal with this problem."

Section 11.2

5. I don't agree that the size of rearranged alleles needs to be routinely reported.

- **Although the committee members generally disagree, we realize that different laboratories and healthcare systems have different needs. As the document is a guideline, rather than a standard, we do not believe that provision for some heterogeneity in practice requires a change in the document itself.**

Related NCCLS Publications*

H43-A **Clinical Applications of Flow Cytometry: Immunophenotyping of Leukemic Cells; Approved Guideline (1998).** This document provides performance guidelines for the immunophenotypic analysis of leukemic and lymphoma cells using immunofluorescence-based flow cytometry; guidelines for sample and instrument quality control; and precautions for data acquisition from leukemic cells.

M29-A2 **Protection of Laboratory Workers from Occupationally Acquired Infections; Approved Guideline—Second Edition (2001).** Based on U.S. regulations, this document provides guidance on the risk of transmission of hepatitis viruses and human immunodeficiency viruses in any laboratory setting; specific precautions for preventing the laboratory transmission of blood-borne infection from laboratory instruments and materials; and recommendations for the management of blood-borne exposure.

MM3-A **Molecular Diagnostic Methods for Infectious Diseases; Approved Guideline (1995).** This document provides guidelines for the use of nucleic acid probes and nucleic acid amplification techniques for the detection of target sequences specific to particular microorganisms. Limitations, quality assurance, proficiency testing, and interpretation of results are also described.

NRSCL8-A **Terminology and Definitions for Use in NCCLS Documents; Approved Guideline (1998).** This document provides standard definitions for use in NCCLS standards and guidelines, and for submitting candidate reference methods and materials to the National Reference System for the Clinical Laboratory (NRSCL).

* Proposed- and tentative-level documents are being advanced through the NCCLS consensus process; therefore, readers should refer to the most recent editions.

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