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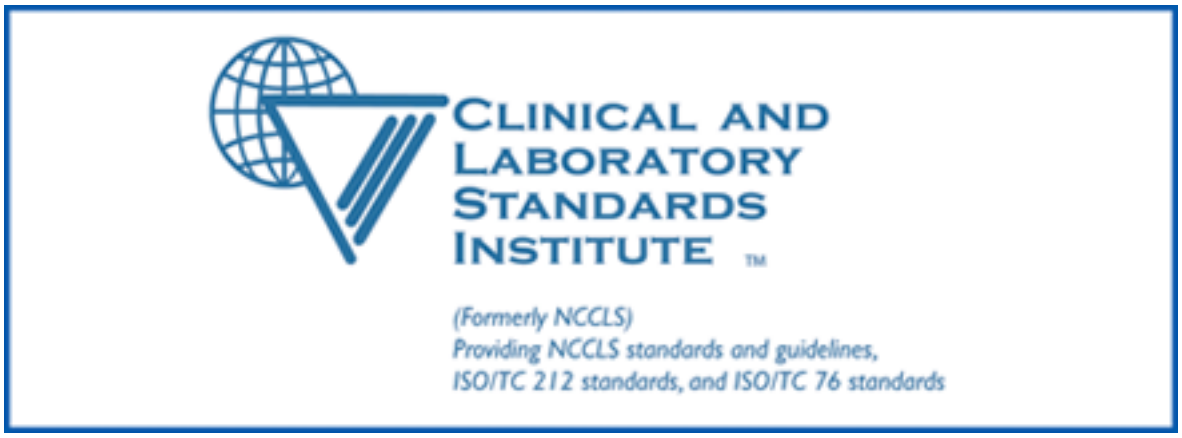
# Nucleic Acid Amplification Assays for Molecular Hematopathology; Approved Guideline




This guideline addresses the performance and application of assays for gene rearrangement and translocations by both polymerase chain reaction (PCR) and reverse-transcriptase polymerase chain reaction (RT-PCR) techniques and includes information on specimen collection, sample preparation, test reporting, test validation, and quality assurance.

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

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## Abstract

The use of gene rearrangement in diagnostic hematopathology is now a standard of practice for which recommendations for assay performance and application have been developed. During the period of development, however, a shift has occurred in the way that gene rearrangement assays are accomplished. Now, many laboratories are performing assays first using polymerase chain reaction (PCR).

Amplification methods, including polymerase chain reaction (PCR) and reverse-transcriptase polymerase chain reaction (RT-PCR), transcription-based amplification (TAS), strand displacement amplification (SDA), ligase chain reaction (LCR), and other methods are now widely used in diagnostic hematopathology. This guideline addresses the needs of the laboratory community by providing guidance on a variety of amplification-based laboratory tests. It addresses preanalytical and analytical factors affecting assay performance, reporting of laboratory results, and quality assurance issues. Immunoglobulin and T-cell receptor gene rearrangement assays, assays for chromosomal translocations, and sequence analysis assays are all considered. The guideline thus provides a basis for laboratory implementation and quality assurance in this important area of diagnostic molecular medicine.

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## Contents

## Contents (Continued)

## Foreword

The Subcommittee on PCR-Based Assays in Molecular Hematology was formed to address the need for a guideline on the performance and interpretation of amplification-based assays in diagnostic hematopathology. Although much of the impetus for this work came from the increasing popularity of PCR-based assays for assessing T-cell receptor and immunoglobulin heavy chain gene rearrangement, the guideline addresses both technical methods and quality control, not only for gene rearrangement methods, but also for assays that involve the detection of point mutations or chromosomal translocations. Although we do not specifically address other types of assay methodologies, we anticipate that this guideline will also be useful in implementing other types of assays (such as fluorescent *in situ* hybridization assays) that are becoming more frequently used in diagnostic hematopathology.

The methods and quality control approaches described herein are not absolute or immutable. They represent formal recommendations presented by the subcommittee, and are intended for use by both manufacturers and diagnostic laboratories. Such use is intended to facilitate both interlaboratory comparisons of results and diagnostic interpretations, as well as to ensure accuracy in diagnosis.

This guideline is written for laboratory directors, surgical pathologists, medical technologists, other laboratory personnel, hematopathologists, hematologists, oncologists, manufacturers of instruments and reagents used in these assays, and those involved in the promulgation of regulations under which laboratories and manufacturers must operate.

**Note that the following trade names are included in NCCLS document MM5-A—*Nucleic Acid Amplification Assays for Molecular Hematopathology; Approved Guideline:* HotStart,® Invader,® LightCycler,® NASBA,® TaqMan,® TMA,™ and SYBR.® It is NCCLS policy to avoid using trade names unless the products identified are the only ones available, or they serve solely as illustrative examples of the procedure, practice, or material described. In this case, the subcommittee and area committee believe the trade names are important descriptive adjuncts to the document. Please include in your comments any information that relates to this aspect of MM5-A.**

**While MM5-A thus describes a number of available laboratory approaches that can be used for laboratory implementation and quality assurance of nucleic acid amplification assays, MM5-A neither requires nor recommends the use of any one specific approach. Since one or more of these technologies and/or procedures described herein may be patented, those who choose to use a specific technology or procedure should determine if a patent exists, and comply with the requirements of the patent holder. Please include in your comments any information that relates to this aspect of MM5-A.**

## Key Words

Amplification, coagulation, gene rearrangement, hematopathology, hemoglobinopathy, immunoglobulin, leukemia, lymphoma, polymerase chain reaction, Southern blot, translocation

# Nucleic Acid Amplification Assays for Molecular Hematopathology; Approved Guideline

## 1 Scope

The use of molecular methods utilizing amplification methods in clinical diagnosis presents new challenges to the pathologist. Despite the clear benefits of having a new method by which to identify monoclonal cell proliferations, chromosomal translocations, the production of abnormal gene messages, or germline mutations of monoclonal cell populations, issues of sensitivity and "false positives" mandate the application of stringent laboratory practice. To assure the success of nucleic acid diagnostics, several key areas warrant attention.

This document addresses the following topics as they relate to direct detection of T- and B- cell gene rearrangements, chromosomal translocations, and germline mutations:

a) indications for molecular biologic testing;

b) specimen collection, transport, and processing;

c) assessment of specimen adequacy;

d) conduct of amplification-based molecular hematology assays; sensitivity, specificity, controls, and artifacts;

e) quality assurance; and

f) interpretation of results.

## 2 Introduction

### 2.1 Diagnostic Utility

The interpretation of biopsies and aspirates in 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 may assist significantly in arriving at the diagnosis of leukemia or lymphoma, or in detecting its recurrence at levels below those discernible to the human observer using a light microscope.

### 2.2 Advantages and Disadvantages

Prudent clinical use of amplification-based molecular laboratory methods to identify monoclonal proliferations of lymphoid cells, to determine the presence or absence of disease-related chromosomal translocations, or to identify gene sequence alterations requires a thorough understanding of the sensitivity and technical artifacts associated with these methods, extreme care in assay performance (to avoid carry-over of amplification products), and the ability to prudently weigh the results, together with clinical findings and histology, to arrive at a diagnosis. To avoid erroneous interpretation, amplification-based hematologic assays require careful attention to technical detail and the implementation of rigorous quality assurance measures.

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Amplification-based assays for immunoglobulin and T-cell receptor gene rearrangements may not identify all possible rearrangements that can be recognized by Southern blotting techniques. Furthermore, the sensitivity for detecting small monoclonal populations may sometimes be lower than that of Southern blotting methods. For these reasons, some laboratories use amplification-based assays only in circumstances in which Southern blotting is unlikely to yield interpretable results, while others reserve Southern blotting for cases in which amplification-based assays are unsuccessful.

This guideline is intended to assist laboratories which rely on nucleic acid amplification-based hematology assay systems to properly implement these techniques, together with the appropriate controls in their laboratories. They are further intended to help the laboratorian determine what types of materials and records must be preserved following the laboratory procedure, and for how long. Finally, they are intended to assist both manufacturers of diagnostic kits and reagents and those responsible for monitoring compliance with quality assurance programs.

# 3 Definitions

**Amplification - 1)** The process of using substances that directly increase signal in proportion to quantity of analyte; **2) Logarithmic amplification,** *n* - Amplification with a response that is logarithmic rather than direct.

**Coagulation** - The process by which the coagulation factors in blood interact to form a clot.

**Immunoglobulin (Ig)** - Any of several classes of structurally related glycoproteins that function as antibodies or receptors and are found in plasma, other body fluids, and in the membranes of certain cells.

**Immunoglobulin class//Immunoglobulin isotype** - A classification of immunoglobins based on antigenic and structural differences of the heavy (H) chain; **NOTE:** There are five classes: IgG, IgA, IgM, IgD, and IgE.

**Polymerase chain reaction (PCR)** - A method for producing multiple copies of a segment of DNA or RNA to test for the presence of the organism of interest.

**Southern blot** - DNA that has been transferred from a gel following electrophoresis and bound to a solid support membrane so that it can be hybridized with a labeled nucleic acid probe.

# 4 Indications for Amplification-Based Molecular Hematology Studies

## 4.1 Indications for Gene Rearrangement Studies

Amplification-based gene rearrangement studies are technically demanding and expensive procedures which should have proper indications if 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. Within these general guidelines, the following may be appropriate uses of immunoglobulin and T-cell antigen receptor gene rearrangement studies:

- to distinguish a reactive (frequently “atypical” histologically) from a malignant lymphoproliferative process in tissue or blood;

- to document organ involvement by a lymphoproliferative disorder (e.g., skin);

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- to facilitate distinction between poorly differentiated nonhematopoietic and "anaplastic" lymphoid neoplasms;

- to detect small amounts of residual or recurrent lymphoproliferative disease in a tissue or body fluid specimen;

- as a general aid in the distinction of acute lymphoblastic leukemia from acute myelogenous leukemia;

- 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;

- as an aid in the distinction of Hodgkin's disease vs. non-Hodgkin's lymphoma (immunoglobulin gene rearrangement is only infrequently detected in Hodgkin's disease); and

- to determine whether two neoplastic biopsies reflect two different disease processes.

Amplification methods are not necessarily more sensitive for the detection of rearranged clones than are Southern blot methods. In general, Southern blot assays are capable of detecting a higher fraction of possible rearrangements than are amplification assays. Furthermore, depending on the specific rearrangement present in a clonal population, Southern-blot-based assays may, in some cases, be able to detect rearrangement involving a smaller fraction of the cellular population than the corresponding amplification assay.

## 4.2 Indications for Identification of Chromosomal Translocations

In general, the indications for using molecular tests to find specific chromosomal translocations are similar to the indications for gene rearrangement studies. Chromosomal translocation studies are most commonly performed to:

- assist in classification of neoplasms characterized by a specific chromosomal rearrangement, such as the t(14; 18) or bcl-2 gene rearrangement of follicular lymphoma (or follicle center cell-derived lymphomas); and

- detect small amounts of residual or recurrent lymphoproliferative disease in a tissue or body fluid specimen.

## 4.3 Indications for the Identification of Point Mutations

Although the detection of point mutations may eventually prove to be useful in the characterization of neoplastic processes, the primary use of gene sequence analysis in diagnostic molecular hematology is to identify sequence abnormalities corresponding to known genetic causes of hematologic disease, such as the Factor V Leiden mutation in patients with thrombosis and activated protein C resistance.

## 4.4 Advantages and Disadvantages of Amplification Assays

Amplification-based gene rearrangement (immunoglobulin, T-cell receptor, and chromosomal translocation) procedures have the following advantages over gene rearrangement assays performed by Southern blot:

- Amplification-based procedures may be accomplished over a time of one to two days, in contrast to one to two weeks for Southern-blot-based procedures.

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- Amplification-based procedures require less material than do Southern-blot-based procedures. As a result, assays may be run on samples such as skin biopsies and fine needle aspirates that would not provide sufficient DNA for a "conventional" Southern blot method.

- Amplification-based procedures may be successfully performed in formalin-fixed, paraffin-based material in a high percentage of cases.

Similar advantages are found for detection of gene sequence abnormalities, whether the assay is based upon the creation or destruction of a restriction site by mutation, allele-specific oligonucleotide hybridization, or complete sequence analysis.

Nevertheless, amplification-based assays may not detect all possible gene rearrangements that can be identified by Southern blotting techniques. Furthermore, the sensitivity for detecting a small monoclonal population may be either higher or lower than that of Southern blotting methods, depending on the particular rearrangement. For these reasons, some laboratories use amplification-based assays only in circumstances in which Southern blotting is unlikely to yield interpretable results.

# 5 Specimen Identification and Accessioning

## 5.1 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 the specimen was acquired.

## 5.2 Requisition Forms

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

- patient name;

- date of birth;

- gender;

- unique identifier found on the specimen container;

- site from which specimen was obtained;

- condition in which specimen was submitted (fresh, frozen, ethanol-fixed, etc.);

- reason for requesting test; and

- referring physician or health professional.

## 5.3 Criteria for Rejecting Specimens

Each laboratory should have written criteria for acceptance or rejection of specimens. Rejection of specimens is strongly recommended if either the specimen or the requisition form lacks sufficient

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information for the laboratory or clinician to uniquely identify the specimen, or other information necessary to determine if the specimen or test requested is likely to be unsuited to the 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 may choose not to accept cerebrospinal fluid, because they receive an insufficient number of specimens of this type to provide certainty that the laboratory has the technical expertise necessary to handle them competently. If a laboratory accepts specimens which are limited in their 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. One cannot obtain a splenectomy specimen twice from the same patient. As with other clinical laboratory procedures, therefore, the ultimate criterion upon which to determine whether or not to perform a molecular assay must be based upon the best interest of the individual patient.

### 5.4 Accessioning Specimens

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

## 6 Recordkeeping, Record Retention, and Specimen Retention

### 6.1 Types of Records

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

- membranes containing bands visualized by colorimetric techniques, such as immunoenzyme methods;
- X-ray film containing autoradiographs of the blot;
- photographic film containing images of bands 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.

Photographs may also be used to create a record of results from “dot blots.”

Manual gene sequencing methods typically result in the production of an image on X-ray film which, if properly labeled, serves as an acceptable record of these results. Semiautomated gene sequencing devices give rise to both an electronic record of the results and, if desired, a record of both band pattern and its

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associated sequence. Other assay formats, such as those produced using fluorometric methods[a] also produce both electronic records and a "hard copy" format.

Production and retention of "hard copy" is recommended, both because of the risk of hardware failure and, more importantly, because changes in technology may render a particular electronic storage format obsolete and unavailable within less than ten years. Such "hard copy" can also be incorporated directly into the laboratory report, if deemed appropriate by the laboratory director and clinical staff.

### 6.2 Identification and Cross-Linking of Records

All patient records, whether paper, film, or electronic, should be permanently labeled with a unique assay identifier which may 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 (such as immunoglobulin heavy chain PCR) and the 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, within each sequencing run, and within each run using any other assay format.

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 this record linkage and any one of the unique identifiers. If computer databases are used for linkage, they should be backed up after the entry of each new batch of patient information.

### 6.3 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 which record the assay results (for example, autoradiographs of blots).

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

## 7 Specimen Transport and Storage

There is limited data on the absolute requirements for transport and storage of tissues for molecular testing. Although the conditions for specimen preservation are less stringent for DNA-based amplification assays than for conventional Southern blot assays, it may be necessary on occasion to perform Southern blot gene rearrangement assays to resolve an inconclusive amplification result. Furthermore, the conditions for preservation of RNA for PCR-based assays are, if anything, more stringent than those required to preserve DNA for Southern blot. For these reasons, the recommendations are to handle tissue for amplification assays with no less care.

When amplification-based methods are used for molecular testing, the risk of obtaining false-positive results because microscopic amounts of tissue from one patient have been admixed with the tissue from another patient is high. Great care must therefore be taken in the process of specimen collection. To the extent possible, specimens should be handled only with disposable instruments, which should then be discarded between specimens. When this is not possible, instruments must be discarded for cleaning after each specimen, and new, clean, and preferably sterilized instruments employed. It is not possible to use

[a] Such as TaqMan® (or equivalent), LightCycler,® or Molecular Beacon Methods.

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too much care to prevent specimen contamination, as the consequences of even "trivial" amounts of contamination may result in incorrect laboratory results and grave iatrogenic consequences for the patient.

## 7.1 Transport and Storage of Solid Tissue

Solid tissue from postmortem or surgical specimens is appropriate for molecular testing. Ideally, tissue should be snap-frozen or fixed immediately. If freezing is not possible, refrigeration may be adequate, even for reverse transcriptase-PCR (RT-PCR) assays, if the specimen can be frozen or minced and placed in a ribonuclease-inhibiting buffer, or if nucleic acid isolation can be begun within an hour. If none of these steps can be accomplished, a portion of the tissue should be fixed. Alcohol-based fixatives are preferred for samples from which nucleic acids are to be isolated. Formaldehyde and other cross-linking fixatives give less optimal results, but may also be utilized successfully provided that the fixation time is not excessive, and that the nucleic acid is assessed for successful amplification of a ubiquitous target such as a housekeeping gene in addition to the gene target of interest. Fixation of the entire tissue sample may, however, render a specimen unacceptable for traditional Southern blot testing, and quite suboptimal for RT-PCR testing.

### 7.1.1 Frozen Tissue

Snap-frozen tissue can be transported on dry ice and stored at $-70 \pm 10\ ^{o}C$.

### 7.1.2 Fresh Tissue

Fresh tissue can be transported on wet ice. Fresh tissue should be frozen, minced, and placed in a ribonuclease-inhibiting buffer; or processed for testing immediately upon arrival in the laboratory.

### 7.1.3 Fixed Tissue

Solid tissue minced in ethanol can be transported and stored at room temperature for periods greater than one year. Solid tissue fixed in formalin and embedded in paraffin may give acceptable results for up to 80 years following removal. Nevertheless, this cannot be guaranteed, and results on formalin-fixed, paraffin-embedded tissue are only obtained at the cost of considerably increased labor, decreased sensitivity, and decreased assay reliability.

## 7.2 Transport and Storage of Thin Sections

Thin sections from paraffin-embedded tissue or frozen blocks are appropriate for PCR-based tests, although paraffin-embedded tissues are suboptimal and result in assays that are less sensitive than Southern blot analysis for detecting both gene rearrangements and chromosomal translocations. Tissue for frozen sections should be frozen immediately after removal. Tissue for paraffin sections should be fixed immediately after removal.

Great care must be taken to avoid carry-over of nucleic acids from one patient specimen to another during the preparation of sections. The use of disposable microtome blades, discarded between specimens, is strongly recommended. These blades may then be sent to the routine histopathology laboratory for further use.

### 7.2.1 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 for years at $-70\ ^{o}C$.

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### 7.2.2 Fixed Sections

Fixed, paraffin-embedded tissue can be stored at room temperature for many years. Paraffin sections that have been cut onto microscope slides should be covered with paraffin to decrease the rate of tissue oxidation. Extremes of temperature and long storage times can decrease the likelihood of successful molecular testing.

## 7.3 Transport and Storage of Cells and Other Fluids

Cultured cell lines, cells from cerebrospinal fluid (CSF), pleural, pericardial, and ascitic fluids, as well as isolated mononuclear cells from blood and bone marrow, are appropriate for molecular testing. In addition, cells from other sources, such as buccal scrapes, may be appropriate for certain types of tests (such as Factor V Leiden testing). 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, viably frozen in dimethylsulfoxide (DMSO), fixed in ethanol, or prepared as cytological cell blocks and embedded in paraffin.

### 7.3.1 Frozen Cells

Cell pellets and frozen cell suspensions should be stored at -70 °C. Pellets and frozen cell suspensions can be transported packed in dry ice. When possible, a ribonuclease inhibitor should be incorporated in the cell suspension medium.

### 7.3.2 Fresh Cells

Cell pellets and fresh cell suspensions can be stored at 4 °C for brief periods of time (less than one hour for RT-PCR assays). For 24-hour storage, cell suspension in a ribonuclease-inhibiting medium is preferable to cell pellets. Cell pellets can be transported on wet ice for rapid receipt. Cell suspensions can be transported on wet ice for overnight delivery.

### 7.3.3 Fixed Cells

Cells that have been fixed may be kept in an alcohol- or acetone-based fixative for prolonged periods of time, but exposure to cross-linking fixatives, such as formalin or glutaraldehyde, rapidly renders cell suspensions unsuitable for molecular testing. Rapid fixation followed by embedding in a paraffin cell block is somewhat superior. Nevertheless, as for tissue specimens (see Section 7.2.2), fixation and embedding results in specimens that are substantially less suitable for molecular testing than specimens that are received fresh or frozen.

## 7.4 Transport and Storage of Whole Blood and Bone Marrow Aspirates

### 7.4.1 Whole Blood

DNA-based testing may be performed on anticoagulated (EDTA or citrate) whole blood that has been stored at room temperature (22 to 25 °C) for up to 24 hours, and may be performed for 72 hours or more on whole blood that has been stored at 2 to 6 °C. Successful RT-PCR analysis requires that endogenous ribonucleases be removed or successfully inhibited within one hour or less after collection. This is accomplished most efficiently by removal of the eosinophilic component. As a specimen ages, RNA and DNA both degrade. RNA degradation proceeds very rapidly, and may make a specimen unsuitable for RT-PCR analysis within one to six hours at room temperature. Although DNA degradation occurs more slowly, prolonged storage at room temperature should be avoided.

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### 7.4.2 Bone Marrow Aspirates

Anticoagulated (EDTA or citrate) bone marrow aspirate should be stored and transported at 4 °C. Although adequate DNA-based molecular results are frequently obtained with aspirates stored and transported at room temperature for up to 24 hours, ribonuclease inhibition or removal within one hour after specimen collection is required to assure the reliability of RT-PCR assays.

# 8 Quality Control

## 8.1 Reagent Quality Control

The complex nature of these procedures coupled with the relatively small number of commercially available reagents and kits makes quality control of reagents particularly important in the laboratory performing nucleic acid amplification.

Buffers and solutions should generally be prepared in advance, given a batch number, and comparatively tested with the previous batch of the same reagent prior to being put into clinical use. Batch numbers should be recorded on the reagent label, and in a reagent logbook which also documents the results of comparative testing, dates that the reagent lot was in use, and dates of preparation and expiration. Expiration dates of certain reagents, such as sterile water, may be listed as not applicable. Under most circumstances, reagents available through a manufacturer should not be used beyond the expiration date set by the manufacturer. In the case of rare reagents that are difficult to source, predetermined procedures and acceptance criteria that are predictive of the quality of the reagent should be documented and used to validate the efficacy of the reagent prior to use. Where permitted by law and the appropriate accrediting agencies, certain valuable reagents may be used beyond their expiration date, provided that comparative testing demonstrates continued acceptable results; these findings should also be recorded in a logbook and approved by the laboratory director. In the United States, this practice is not currently permitted.

## 8.2 Procedural Quality Control

For each specimen, quality assurance records should be generated and kept on record.

- The sample and requisition should be examined and deemed acceptable, and the condition of the sample upon receipt noted.

- Samples being accepted for testing despite failure to meet the written criteria should be accepted with notation explaining the decision by the laboratory director.

- Records of the sample preparation procedure, DNA quality and yield, (if possible), components of the PCR assay, amplification parameters, and product detection methods should be detailed in a procedural log.

- Performance standards should be determined and recorded for each critical step in the PCR assay. Assay worksheets can include recorded data on the results of sample preparation, temperatures of water baths, times of incubations, results of control amplification, and other such parameters which are crucial parts of the analytical procedure.

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## 8.3 Carry-Over Prevention

### 8.3.1 Spatial Separation of Work Areas

Spatial separation of work areas is imperative for the proper performance of PCR and other nucleic acid amplification methods.

The reagent preparation area is the "cleanest" of the work areas for PCR and can consist of a separate room, a hood with the fan turned off to minimize aerosolization, or an enclosed countertop hood or box, preferably with a UV light inside. This area is used for the preparation of the "master mix" containing buffer, nucleotides, primers, Taq enzyme, and magnesium chloride. Patient samples, prepared DNA, and most importantly, amplified products must never enter this area in order to minimize the risk of contamination leading to a false-positive PCR assay. Since equipment can become contaminated with DNA, it is imperative that dedicated equipment be used and stored within the area. Reagents should be stored in a refrigerator free of DNA (and especially amplicons), and disposables such as tubes, tips, and gloves should also be stored in a manner to prevent their contamination. A clean laboratory coat designated for Area 1 should be used, clean gloves should always be donned, and care should be taken to leave transportables such as pens, tape, and scissors in their designated areas.

The second area is for specimen preparation, including DNA and RNA isolation. It is here that tubes containing the aliquotted master mix should be brought for the addition of DNA; tubes should not then be returned to Area 1. Area 2 activities can be performed in the open laboratory on a bench but might also be performed in an enclosed space, such as a bench-top hood or containment box.

After amplification, reaction tubes should be taken unopened to a third area for product detection. This is the dirtiest of the PCR work areas, and extreme caution must be taken to ensure that laboratory coats, gloves, equipment, tube racks, etc., never move retrograde into cleaner areas.

### 8.3.2 Unidirectional Work Flow

Unidirectional workflow (the avoidance of returning to a preamplification area after working in a postamplification area) is also helpful in minimizing the risk of carry-over, since amplicons can be carried on clothing, hair, and skin. Large laboratories can make bench assignments with separate technologists performing product detection, sample preparation, and PCR set-up. In a small laboratory, however, this becomes much more difficult, and the staff will have to plan their schedules carefully, attempting to avoid the performance of product detection followed by PCR setup in the same day, and above all, be meticulous about their movements around the laboratory.

### 8.3.3 Chemical and Physical Methods to Prevent Amplicon Carry-Over

Chemical means of amplicon carry-over prevention exist, and should be included in the assay design whenever possible. These include the incorporation of UTP in place of dTTP into amplicons, which makes them susceptible to destruction by uracil N-glycosylase and heat, and cross-linking agents such as psoralens compounds. These methods will help to prevent carry-over; they will, of course, not be effective in eradicating contamination of samples with unamplified DNA or organisms during the sample preparation procedure. UV light is partially effective for the destruction of DNA on surfaces, and can be used, along with meticulous clean-up procedures, to help decontaminate work areas.

## 8.4 Positive and Negative Procedure Controls

Controls are a crucial indicator of the success of assay performance. At least one and possibly several negative controls should be included in each assay run to reveal any carry-over or contamination that could be present; at least one of these should be saline or water that is carried through the entire sample

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preparation procedure to assess the potential for contamination prior to the PCR setup. Negative controls should be dispersed among the patient specimens, with one placed at the end of the run. Positive controls should be included, preferably at a reasonable dilution of the target into a biological fluid and should also be carried through the sample preparation method to demonstrate the success of the entire procedure. An internal control target is also important in assessing the success of PCR assays. Such a control, such as amplification of beta-globin or another cellular target, demonstrates the presence of amplifiable DNA in the prepared sample; a positive signal for this control is necessary, particularly to be certain of a negative result for the target of the assay.

For RT-PCR assays targeting messenger RNA, successful amplification of an endogenous mRNA transcript such as a housekeeping gene can be used to demonstrate both the quantity of mRNA and the absence of inhibitors. In this case, primers should be chosen to anneal in different exons so that amplification from mRNA can be differentiated from that generated by any DNA that might be present in the sample.

## 8.5 Equipment Maintenance

All equipment used in these procedures should be maintained and calibrated according to the manufacturers' standards and other applicable NCCLS standards and guidelines. All balances, pipets, spectrophotometers, electrophoresis power supplies, incubators, etc. should be within the calibration due dates.

## 8.6 Quality Assurance Procedures and Records

### 8.6.1 Procedure Manual

Within the laboratory, a procedure manual should be available which 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, procedural quality control records, and equipment quality control records should be defined.

### 8.6.2 Personnel Standards

The procedure manual should indicate the level of education, training, and experience required for individuals performing any of the assay procedures within the laboratory. Special care should be taken to define the requirements for personnel empowered to review the final laboratory test results and release them from the laboratory ("sign-out"). The procedure manual should define the location in which personnel qualification statements are kept, and define methods by which personnel qualifications review/performance evaluations are documented.

## 8.7 Proficiency Testing

Laboratories performing clinical molecular biologic assays should enroll in proficiency testing programs for each assay performed, when such programs are available. Proficiency testing programs may not be available for all tests, however. In these cases, it may be possible to utilize samples from another, closely related program. As an alternative, laboratories may exchange samples with one or more other laboratories performing the same type of test.

# 9 Sample Preparation and Processing

A number of methods exist for the preparation of nucleic acid samples for molecular genetics analysis. Since this step can have a significant impact on the quality of the final results, care should be exercised to

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ensure that an accepted protocol is followed, whether the test method was developed in-house, obtained from a literature review, or purchased from a reputable manufacturer. Protocols that have been either developed in-house or obtained from literature reviews should be validated. Regulatory agency-cleared kits and manufacturer's kits whose protocols are altered in any way should also be validated by the laboratory.

While many of the methods will differ in terms of their protocols and materials used, it is important that, regardless of the procedure used, the integrity of the nucleic acid in the clinical sample be maintained and that impurities present in the final sample preparation be diluted sufficiently to eliminate interference with the test.

Written procedures for the methods used, including the sources for all components used for DNA and/or RNA purification, should be kept. Changes in any of the procedures or source of components should be documented and approved by the laboratory director, with date and initials recorded. For more guidelines on design, preparation, and maintenance of technical procedure manuals, see the most current edition of NCCLS document GP2—*Clinical Laboratory Technical Procedure Manuals.*

## 9.1 Target Nucleic Acid Release

### 9.1.1 DNA Isolation

Traditional methods of DNA isolation from samples generally require the lysis of cells and addition of a proteolytic enzyme such as proteinase K to digest proteins that may bind the DNA and interfere with the procedure. Following this, the samples are usually treated with an enzyme to remove residual RNA and extracted with organic solvents to remove remaining proteins and cell components that can interfere with the quality of the result. Finally, the sample is treated with ethanol to precipitate the purified DNA from the remaining material. Purified DNA may be recovered at this point either by centrifugation or by careful spooling with a glass stirring rod to gather the adhering precipitated DNA. Following this, the purified DNA should be resuspended in a buffer, such as Tris buffer. Excessive drying should be avoided, as it can affect the structural integrity of the purified DNA.

A common modification of this procedure involves the use of a high-salt solution and isopropanol to promote precipitation of the DNA from solution.

Several commercial nucleic acid isolation kits are available that may simplify and reduce the time of this procedure. A variety of methods using different components and procedures to perform the above steps are used. Many of the kits use alternatives for the extraction and precipitation of sample by replacing the organic solvents and ethanol precipitations with other suitable components that are less toxic.

Each laboratory should evaluate its DNA purification system for yield efficiency, consistency, and DNA purity before incorporation into the routine workload.

### 9.1.2 DNA Isolation for Use With Amplification Procedures

The requirements for DNA preparations used for PCR and other amplification methods are generally less demanding than those for Southern analysis. While the more highly purified DNA may be used for these procedures, it is often convenient and cost-effective to use one of the many short procedures found in the literature or one of the commercially available quick-prep kits.

In general, short methods start with small volumes of blood or other cells from a variety of tissue origins. In most cases the cells are lysed with a proteinase K-based buffer, or with alkali, or with a Tris buffer or water and high heat. The resultant lysate is neutralized and cellular debris removed by centrifugation. The

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resulting crude lysate may be used directly in amplification procedures. However, if poor amplification is seen with housekeeping gene primers, further purification may be necessary.

### 9.1.3 RNA Isolation

Clinical assays in which the molecular target is RNA generally give the best results when the RNA is purified. Several protocols and solvent systems are available for the isolation and purification of RNA. As with the isolation of DNA, numerous methods have been reported in the literature, and commercial products are available from a number of different companies. RNA is very sensitive to degradation by RNases which may contaminate glassware and plasticware and enzyme and protein preparations. Extra care is necessary to ensure that all reagents and supplies used in the extraction and storage of RNA are properly treated to destroy or inhibit RNases. Most liquid reagents prepared in the laboratory may be treated with diethylpyrocarbonate (DEPC) followed by autoclaving (exceptions include reagents containing Tris buffer and reagents with heat-sensitive ingredients such as protein and volatile compounds). A variety of commercial preparations are available that either inhibit or destroy this enzyme activity. As with all procedures, the laboratory should carefully follow the established protocol and incorporate established controls to verify proper performance for each extraction.

## 9.2 Quality and Quantity of Nucleic Acid

For tests that will require Southern analysis, the quality and quantity of the DNA obtained should be measured by one of the established methods.

### 9.2.1 Spectrophotometry

DNA can be quantitated using optical density (OD) on a spectrophotometer that has been properly calibrated and with the use of proper controls. To determine the quantity of purified DNA, absorbance readings at 260 and 280 nm should be performed on diluted samples. The determinations should be performed in clean, dry, quartz cuvettes within the linear range of the particular spectrophotometer being used. When making dilutions of concentrated DNA, the difficulty in "dissolving" small portions of concentrated DNA in water or buffer should be appreciated. Resuspension of the DNA aliquot will require heat, mixing, and time to achieve a uniform suspension.

Nucleic acids absorb maximally at 260 nm and proteins maximally at the 280 nm. The RNA concentration can be determined based on the fact that each OD unit at 260 nm is equivalent to 40 μg/mL of single-stranded RNA. An OD $A_{260}/A_{280}$ ratio provides a qualitative measurement of the level of RNA with respect to the amount of contaminating protein that exists in the sample. Ratios above 1.6 indicate high levels of RNA purity. Purity may be improved by re-extraction and precipitation when ratios obtained are below 1.6. RNA with a ratio between 1.6 and 1.9 is often adequate from RT-PCR and other amplification approaches. Human leukocytes generally yield 10 to 40 μg of total RNA per $10^6$ cells.

For amplification procedures, it is possible to use samples for which the $A_{260}/A_{280}$ ratio is below the aforementioned guideline. Appropriate controls should be tested in this type of analysis to determine the suitability of the DNA for such a test.

### 9.2.2 Gel Electrophoresis

The gel electrophoresis method can be used by incorporating undigested lambda or other equivalent marker DNA to determine the proportion of DNA that is high molecular weight. The laboratory should document the parameters which are used to determine acceptable quality. A set of concentration standards consisting of lambda or other equivalent marker DNA can be included for estimation of yield.

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### 9.2.3 Fluorometry

Fluorometric assays should be able to detect double-stranded DNA and should utilize the appropriate human DNA standards. Such assays can be obtained from several commercial vendors and should be used in conjunction with sophisticated instrumentation. It is important that the user carefully follows the manufacturer's protocol for both the assay and the use of the instrument, and includes proper controls to verify performance of the process.

## 9.3 Storage of Nucleic Acids

Isolated DNA and RNA should be stored in tightly capped containers in either nuclease-free water or TE (Tris-EDTA) buffer. Long-term storage should be carried out at –20 °C (DNA) or –70 °C (DNA or RNA) to prevent degradation. Long-term storage of RNA can be improved by storing as an ethanol precipitate. Stability of the sample can be maintained for several months by storing at 4 °C in tightly capped containers. Integrity of samples should be reevaluated before use if stored for extended periods of time at any temperature.

## 9.4 Inhibitors and Interfering Substances

A variety of components within clinical specimens may interfere with enzymatic reactions employed in amplification and detection methods. Heme (at 0.8 mmol/L) and its by-products, along with certain components of sputum glycoproteins, are known to be inhibitors of DNA polymerases. It has also been reported that the actions of certain restriction enzymes may be impeded by heparin.

Precautions should be taken in the method used for DNA preparation, since some components such as EDTA, and certain detergents like SDS may also inhibit amplification enzymes if they copurify with the target nucleic acid.

In general, restriction enzyme digestion of sample for time periods longer than that recommended by the manufacturer does not cause problems. Contamination of the enzyme by exonucleases or DNase can degrade the sample; however, if the enzyme source is from a reputable commercial company, such a problem is rare. It is possible to stop the action of the restriction enzyme by one of three methods: 1) the addition of the sample onto an agarose gel with concentrated loading dyes; 2) freezing the sample to between -16 °C and -24 °C; or 3) adding 0.5 M EDTA with a pH of 7.5 to a final concentration of 10 mM.

### 9.4.1 Detection of Inhibitors and Interfering Substances

Inhibitors of amplification can be detected by the introduction of control templates. Such a control template, if warranted, can be added during sample preparation, thus also serving as a control for the extraction process. The need for use of a control for inhibition should be determined on a case-by-case basis, because development and implementation of such controls is technically complex and can affect assay performance. If, during product development or test evaluation, it is determined that inhibitors or interfering substances are encountered only rarely, it probably is not necessary to run controls for inhibition as described in the following sections. On the other hand, if reaction inhibition is a significant source of false-negative results, such that the assay sensitivity falls below clinically significant levels, controls for inhibition should be strongly considered.

#### 9.4.1.1 Homologous Internal Control

Homologous controls contain the same target sequences as the authentic target, but they are distinguishable from the target by differences in size or by the presence of internal sequences.

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Homologous controls are thus amplified with the same primers used to amplify the target. Homologous controls can be produced in a variety of ways:

- amplification of unrelated sequences with primers containing "tails" that are homologous to the target sequence;
- cloning of the target sequence into a plasmid vector and alteration of the insert to change the electrophoretic mobility or internal sequences of the product; and
- replacement of target sequences (except those homologous to the primers) with unrelated sequences by restriction enzyme digestion and ligation.

9.4.1.2 Heterologous Controls

Heterologous controls do not contain the target sequence, and they require a separate set of primers for detection of control sequences. Heterologous controls commonly used include the human HLA-DQA1 and human ß-globin loci, but any target will generally suffice. The control should be of equal or greater size than the authentic target to ensure that any product of lesser size can be successfully amplified.

9.4.1.3 Implementation of an Internal Control

Control template can be added to the master mix in an amount that will provide 10 to 1,000 copies for each reaction, and should be amplified with homologous or heterologous primers. Care must be taken to ensure that the presence of the internal control does not reduce the sensitivity of the amplification system. A reduction in sensitivity will often occur if the coamplified internal control template is present in too great a quantity.

### 9.4.2 Inhibitory Samples

If the sample will not support the amplification of the internal control, it is unlikely that it will support the amplification of the target nucleic acid sequence. The specimen should be reported as inadequate for analysis by nucleic amplification methods, or it should be further processed to reduce or eliminate the inhibitors. Often a simple dilution of the specimen will reduce the effect of the inhibitor. However, care must be taken not to dilute the specimen so much that the target is diluted beyond the limit of detection.

## 10 Diagnostic Problems in Molecular Hematology—General Considerations

Table 1 summarizes examples of types of molecular abnormalities that are commonly detected in molecular diagnostic methods for hematologic disorders. For all kinds of mutational alteration, direct sequence determination is the gold standard on which other methods are grounded, or used to determine the actual change where other methods indicate a variation.

### 10.1 Deletion Detection

Deletions are detected most simply by amplification with primers that span the region in question and will faithfully amplify whether the deletion is present or not. Alternatively, primers can be designed to amplify or not depending on the presence of a specific known deletion. Deletions are most readily detected as an alteration in the size of the PCR product, (as detected by electrophoresis), or by sequence analysis.

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**Table 1. Types of Molecular Abnormalities Commonly Detected in Molecular Diagnostic Methods for Hematologic Disorders**

| Molecular Abnormality | Examples | Technology to Diagnose |
|---|---|---|
| Deletions or Insertions | α - Thalassemia | PCR, LCR, ARMS, etc. |
| Known Point Mutations and Small Deletions | Sickle Cell Anemia<br>ß- Thalassemia<br>Factor V Leiden | ASO/PCR<br>Allele-Specific LCR<br>PCR-RFLP<br>Dot Blot Sequencing |
| Short Tandem Repeats<br>Triplet Repeats | Linkage in General | PCR/Denaturing Gel Electrophoresis |
| Unknown or Not Yet Fully Characterizable | Hemophilia | RFLP or STR and Linkage Analysis<br>SSCP or DGGE |
| Translocation | Follicular Lymphoma<br>Mantle Cell Lymphoma<br>Chronic Myelogenous<br>Acute Promyelocytic Leukemia | PCR, RT-PCR |
| Clonality | B- and T-cell Non-Hodgkin's Lymphoma and Leukemia | PCR |

## 10.2 Point Mutation Detection

Point mutations are detected most simply with amplification by primers that include the region in question and will faithfully amplify whether the mutation is present or not. These, too, can be multiplexed to contain several mutations of interest. Alternatively, primers can be designed to amplify or not depending on the presence of a specific known mutation (e.g., amplification refractory mutation system [ARMS], or allele-specific amplification, [ASA]) and detected as described above.

If mutation selective amplification is not used, results can then be detected by dot blot or reverse dot blot. Dot blotting is done by applying amplified products to a membrane such as nitrocellulose or nylon (laid out in an array), and testing for the presence of mutant or normal alleles by hybridization with oligonucleotides which are sequence-specific (allele-specific oligonucleotides [ASO]). The detection is usually via radioactively labeled ASOs and autoradiography. Known internal controls can be included in the array, and membranes can be successively probed with different ASOs. With reverse dot blot, the ASOs are laid out on the array and hybridized with patient amplification products. Detection can be with radioactivity or by other kinds of labels in one of the primer pairs as described above. More elaborate arrays with a hundred or more sites, and miniaturization with the use of silicon or glass instead of membranes are coming into use.

If the site of the point mutation affects an endonuclease—either abrogating a site which is present in the wild-type state (e.g., Mst II in sickle cell anemia), or creating a new recognition site—the amplified product may be cleaved with the restriction endonuclease prior to size separation on a gel. This procedure allows the simple and rapid differentiation of homozygous wild-type, homozygous mutant, and heterozygote carrier.

For example, after Mst II digestion of the beta globin PCR product, a single amplified fragment is seen in homozygous (HbSS) sickle cell disease, three fragments are seen in heterozygous (HbAS) sickle cell trait, and two fragments are seen in those without the sickle mutation.

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## 10.3 Short Tandem Repeats and Triplet Repeats

Short tandem repeats (STRs) are structures which occur at defined locations in the genome and are used as landmarks for linkage analysis or, when natural polymorphisms occur in the population, for identity analysis. Triplet repeats are structures which occur in normal alleles, but the number of repeats is greatly increased in the pathological state. In either case, specific amplified products are run out on "sequencing gels," since sequencing gels give high resolution over a suitable range of molecular weights and permit the resolution of different repeating units.

## 10.4 Unknown or Not-Yet-Sequenced Mutations

### 10.4.1 Genetic Linkage Analysis

Linkage analysis is based on the principle of cosegregation of closely linked loci. The closer the loci, the more likely they are to be inherited together. If polymorphic DNA markers are linked to a disease-causing gene, but the gene has not been isolated, genetic linkage analysis can be used in a family in which a mutant allele of the gene is segregating to determine the probability that the mutation was inherited by a particular family member. Alternatively, linkage analysis is useful in prenatal diagnosis of diseases resulting from mutations in genes that are too large for practical direct sequence analysis. Hence, molecular diagnosis of hemophilia A, which results from abnormalities in the 186 kb Factor 8 gene, is most frequently made by RFLP methods.

As an indirect method of mutation detection, linkage analysis has two primary disadvantages. One is the requirement for a family study. Specimens should be collected from multiple family members in multiple generations, and the polymorphic markers should be present in the heterozygous state in certain family members for the results to be informative. The other primary disadvantage is that linkage analysis determines a risk that the disease-causing gene has been inherited, whereas, the direct methods provide a definitive result. For these reasons, linkage analysis is usually considered an interim procedure to be replaced by methods that detect the mutant allele(s) directly.

More extensive discussion of linkage analysis methods is found in the most current edition of NCCLS document MM1—*Molecular Diagnostic Methods for Genetic Diseases.*

### 10.4.2 Mutation Scanning Methods

Mutation scanning methods are applicable after a gene has been isolated and at least partially characterized. These methods are used to "scan" the gene, usually the coding sequences, for the purpose of narrowing down the location of a mutation or polymorphism to a relatively small segment, usually no larger than an exon. Once the segment containing the mutation has been identified, its exact nature and location can be determined by sequencing. This approach obviates sequencing a larger portion of the gene to find a specific mutation. Although in theory some of the scanning methods should be capable of detecting essentially 100% of mutations, in practice the percentage detected is usually lower, and in some cases, much lower.

Mutation screening methods can be useful in the following situations: a) when a disease-causing gene has been isolated and the exons sequenced, but few mutations have been identified; b) when the gene in question has several high-frequency mutant alleles, but none, or only one of the expected two, were detected in the patient's specimen by direct detection methods; and c) when a gene has many known mutant alleles, all occurring at low frequency.

β-Thalassemia provides a typical example of a hematologic disease for which mutation scanning (most frequently denaturing gradient gel electrophoresis) is most commonly employed.[1] In general, successful use of mutation scanning methods requires that a relatively small number of mutations account for most

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disease; when mutations are more evenly distributed throughout a gene, RFLP methods are likely to prove more robust.

Several commonly used mutation-scanning methods are described below. All of these, except single-stranded conformational polymorphism analysis, are based on differences between a homoduplex and the corresponding heteroduplex. All of them utilize a target amplification method, most commonly PCR.

#### 10.4.2.1 Denaturing Gradient Gel Electrophoresis (DGGE)

The basic principle of DGGE is that DNA duplexes migrate through a gradient of denaturant until they reach a position where the strands melt. At this point, no further migration occurs. The melting behavior of a double-stranded DNA molecule is a function of the base composition of its constituent sequences. When such a sequence is altered (as in the case with mutant/wild-type DNA heteroduplexes), so is its melting behavior and electrophoretic migration.

A double-stranded DNA molecule actually consists of melting domains. The AT-rich domains ("early-melting") begin to melt in a lower concentration of denaturant (or at a lower temperature). The GC-rich domains ("late-melting") melt last, at a higher concentration of denaturant (or a higher temperature). DGGE is relatively insensitive to mutations located in late-melting domains. To overcome this problem, most techniques have now incorporated a "GC-clamp," which serves to stabilize the duplex and permit detection of differences in the melting profile of the remainder of the fragment.

#### 10.4.2.2 Chemical Mismatch Cleavage (CMC)

The basic principle of CMC is that mismatched base pairs in hybrid duplexes formed between wild-type and mutant DNA are susceptible to chemical modification and cleavage. Briefly, the procedure is carried out as follows: each PCR product derived from the patient's mutant gene is denatured and reannealed to a labeled, homologous, normal PCR product. Mispaired C or T residues in the heteroduplexes formed are then modified by hydroxylamine (for C mismatches) and osmium tetroxide (for C and T mismatches). The sites of the mismatches are then cleaved by treatment with piperidine, and reaction products are detected after gel electrophoresis. The relative sizes of the reaction products provide some indications of the location of the lesions within a given DNA fragment. In principle, CMC detects all types and classes of mutation. However, since mismatched G and A bases cannot be detected directly, but only indirectly through C and T mismatches, both sense and antisense strands should be tested.

#### 10.4.2.3 RNase A Cleavage

Mismatches in RNA:DNA hybrids or RNA:RNA hybrids are detectable by RNase A cleavage of the single-stranded (mismatched) region. The labeled RNA of known sequence (usually normal) is mixed with double-stranded target DNA from a patient, heated to denature, reannealed, and treated with RNase A. The RNase A removes the 5' and 3' overhangs and cleaves the RNA at any single-stranded site in the RNA:DNA hybrid. Denaturing gel electrophoresis separates the RNA fragments according to size. Mutations are detected by loss of the wild-type band and the appearance of two smaller bands.

#### 10.4.2.4 Heteroduplex Analysis

All of the methods described above are types of heteroduplex analysis. But, in the United States, at least, one specific form of heteroduplex analysis has come to be known by this term. This relatively simple method is based on the principle that heteroduplex DNA usually has a slower electrophoretic migration rate than either corresponding homoduplex.

The region to be tested for the presence of a mutation (or sequence polymorphism) is first amplified by PCR. (If the target DNA is present as two different alleles, heteroduplex DNA will form during PCR

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cycling.) In practice, the resulting PCR product is mixed with PCR product generated from normal target. The mixture is denatured, reannealed, and then separated by electrophoresis in acrylamide gel or in a modified polyacrylamide-based vinyl polymer. If the mixed PCR products represent different alleles (normal and mutant), four distinct species will be generated: wild-type homoduplex, mutant homoduplex, and two different heteroduplexes. Often these different species can be separated by electrophoresis.

A major advantage of heteroduplex analysis is its simplicity. A drawback is the size limitation. The efficiency of mutation detection is much lower for longer PCR products, and drops off drastically above 350 to 370 base pairs, depending on the type of mutation being detected. Generally, insertions and deletions are more easily detected than single base-pair replacements.

10.4.2.5 Single-Stranded Conformational Polymorphism (SSCP) Analysis

The principle of SSCP is that the electrophoretic mobility of a single-stranded DNA molecule is a function of nucleotide sequence, as well as length. Both parameters determine the overall shape, effective size, and surface charge density of a single-stranded DNA molecule. Thus, in many cases, single-stranded PCR products differing by only a single nucleotide can be distinguished.

In practice, PCR products are denatured and run on polyacrylamide gels under nondenaturing conditions. In theory, this allows each single strand to fold upon itself. If two strands differ by even a single nucleotide, potentially they will fold differently, if conditions are appropriate. Thus, the strands will assume slightly differently shapes and effective sizes and, hence, will migrate at different rates during electrophoresis.

# 11 Nucleic Acid Amplification Technologies

## 11.1 Amplification Systems

The need for more sensitive nucleic acid-based tests and the desire to replace radioisotopically labeled probes was met with the introduction of nucleic acid amplification followed by detection. In the past, nucleic acid probe tests relied upon the use of radioisotopes to attain maximum sensitivity. However, the ability to amplify target DNA from one copy to millions increased the sensitivity of these tests. Coupled with the introduction of more sensitive nonradiometric detection systems (e.g., chemiluminescent probes), the potential of achieving excellent test sensitivity without the hazards of radioactivity became possible.

Nucleic acid amplification began with the development of the polymerase chain reaction (PCR) and the use of a patented thermostable polymerase (i.e., *T. aquaticus* (*Taq*) DNA polymerase). Following the introduction of PCR, a multitude of nucleic acid amplification systems was described (see Table 2). These can be divided into three amplification types: target, probe, and signal. A crucial issue with any of these ultrasensitive tests is avoiding carry-over product contamination. All amplification techniques require containment protocols to effectively control contamination. For example, PCR may also be performed with modified bases (i.e., uracil) during amplification. These may be specifically destroyed before subsequent amplifications (i.e., with uracil-N-glycosylase [UNG]), hence eliminating any crossover contamination from previous amplification reactions.

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**Table 2. Amplification Methods**

| Test | Amplification Type | Nucleic Acid Target | Enzymes Needed | Thermal Cycling Needed | Applications |
|---|---|---|---|---|---|
| PCR | Target | DNA (RNA)* | DNA polymerase (thermostable) | Yes | Organism/gene detection<br>Sequencing<br>Cloning |
| TAS | Target | DNA/RNA | Reverse transcriptase<br>RNA polymerase | No | Organism/gene detection<br>Sequencing<br>Cloning |
| 3SR | Target | RNA (DNA)† | Reverse transcriptase<br>RNA polymerase<br>RNase H | No | Organism/gene detection<br>Sequencing<br>Cloning |
| SDA | Target | DNA† (RNA)* [2] | Hinc II<br>DNA polymerase I (Exonuclease deficient)<br>Bst polymerase<br>BsoB1 restriction endonuclease<br>Reverse transcriptase | No | Organism/gene detection<br>Sequencing<br>Cloning |
| NASBA®/ TMA™ | Target | RNA (DNA)† | Reverse transcriptase<br>RNA polymerase<br>RNase H | No | Organism/gene detection<br>Sequencing<br>Cloning |
| QBR | Probe | DNA/RNA | Q-beta replicase | No | Organism/gene detection |
| LCR | Probe | DNA | DNA ligase (thermostable) | Yes | Organism/gene detection |
| GapLCR | Probe | DNA | DNA ligase (thermostable)<br>DNA polymerase (thermostable) | Yes | Organism/gene detection |
| bDNA | Signal | DNA/RNA | Alkaline phosphatase | No | Organism/gene detection |

* Reverse transcriptase can be used to convert RNA to DNA before PCR.
† DNA must be denatured before amplification.

PCR polymerase chain reaction
TAS transcription-based amplification
3SR self-sustained sequence replication
SDA strand displacement amplification
NASBA® nucleic acid sequence-based amplification
QBR Q-beta replicase
LCR ligase chain reaction
bDNA branched DNA
DNA deoxyribonucleic acid
RNA ribonucleic acid


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### 11.1.1 Target (PCR, TAS, 3SR, SDA, and NASBA®) Amplification

All target amplification methods rely upon an initial specific base pairing of oligonucleotide(s) (i.e., primer or probe) with a target nucleic acid (DNA or RNA). The major differences between these methods lie in the amplification scheme, the type and number of enzymes used in the reaction, and whether temperature cycling is required. Total reaction time for the amplification process also varies widely between the different methods.

Polymerase chain reaction (PCR) technology uses a thermostable DNA polymerase to synthesize a region of target DNA defined at each end by a specific primer (Figure A1 in the Appendix). The primer pair consists of short oligonucleotides (15 to 30 bases) chosen from known nucleic acid sequence of the target and corresponding-to-complementary DNA strands. In theory, the reaction is capable of producing billions of copies of target DNA from a single starting copy. The reaction is accomplished by a series of temperature changes and consists of three steps: denaturation (DNA strand separation at high temperature); annealing (primer attachment to a complementary strand at a lower but still stringent temperature); and extension (5' to 3' DNA strand synthesis starting with the primer at a moderate temperature). The annealing and extension steps can also be combined into a single step. These two to three steps define a cycle. Theoretically, there is a doubling of the target sequence at each cycle (i.e., $2^n$, where $n$ = cycle number). Usually 25 to 40 cycles are performed followed by product detection. RNA can also be used as a target following an initial synthesis of a complementary DNA (cDNA) strand by reverse transcriptase (RT-PCR).

A transcription-based amplification system (TAS) is an *in vitro* RNA transcription system that uses reverse transcription to produce cDNA (i.e., via a reverse transcriptase) and RNA transcription (i.e., via an RNA polymerase) to produce RNA. The primers contain a promoter sequence for a DNA-dependent RNA polymerase at one end and a specific target sequence at the other end. Thus, target amplification is the result of many RNA molecules being produced from each cDNA template. A disadvantage of TAS is that it is carried out at moderate temperatures (e.g., 37 °C and 42 °C), which may result in nonspecific primer hybridization and decreased specificity.

Self-sustained sequence replication (3SR) and nucleic acid sequence-based amplification (NASBA®) are similar. 3SR is basically a modification of TAS. The 3SR scheme (Figure A2 in the Appendix) is isothermal and introduces an additional enzyme, RNase H. The RNAse H digests RNA from the intermediate RNA–DNA hybrid so that complete cDNA synthesis can occur. Starting with RNA, 3SR basically simulates the retroviral replication cycle *in vitro*. However, DNA can also be used if a denaturation step is introduced before the 3SR reaction.

Strand displacement amplification (SDA) (Figure A3 in the Appendix) is an isothermal, *in vitro* DNA amplification technique that is based on the ability of a restriction enzyme to nick the unmodified strand of a hemiphosphorothioate form of its recognition site, and the ability of a DNA polymerase to initiate replication at the nick and displace the downstream nontemplate strand. SDA primers containing recognition sites for the nicking restriction enzyme bind to opposite strands of previously heat-denatured target DNA at positions flanking the sequence to be amplified and are extended. Amplification template strands (target fragment) containing the restriction enzyme recognition site are displaced from the starting target by extension of bumper primers that bind upstream to the SDA primer binding sites. The target fragment is exponentially amplified under isothermal conditions by coupling sense and antisense reactions in which strands displaced from the sense reaction serve as a target for the antisense reaction and vice versa.

### 11.1.2 Probe Amplification

The principle of the Q-beta replicase scheme (Figure A4 in the Appendix) is that a modified RNA template (i.e., MDV-1), containing sequences complementary to the target, hybridizes specifically to the

target and can subsequently be amplified to billions of copies. Q-beta replicase is a unique RNA polymerase that does not require a primer to initiate RNA synthesis. Since no primers are included in the reaction, the specificity lies in the hybridization of the probe sequence inserted into the MDV-1 molecule to the target DNA or RNA. Following hybridization and removal of unhybridized MDV probes, an amplification step is performed in which the MDV probes are replicated up to extremely high levels. These can be detected directly (e.g., by isotope incorporation or fluorescence), in which case Q-beta replicase techniques can be considered a signal amplification method. Alternatively, because the probe sequence, in most cases, is amplified along with the MDV sequence, Q-beta amplification could loosely be called a target amplification method. Its unique properties probably justify coining a new term, "probe amplification." This term would also encompass other hybrid methods such as ligase chain reaction (LCR).

LCR is the trademark name for ligation amplification reaction (LAR). The test (Figure A5 in the Appendix) uses a thermostable DNA ligase to link two adjacent oligonucleotide probes on each DNA strand of the target (i.e., four probes in total). Similar to PCR, the reaction uses temperature cycles to denature and anneal. In contrast to PCR, LCR uses ligation in place of extension to form a product. The product then serves as a target for subsequent reactions. Biotinylated probes can be used to simplify and expedite the detection process. A variant of LCR, gap-LCR, introduces a gap between the sequences to which the probes anneal. This gap is then filled in with a thermostable polymerase, and the probes are then ligated.

### 11.1.3 Signal Amplification

RNA or DNA is detected in a sample based on a unique solution-phase sandwich hybridization assay coupled with signal amplification employing branched DNA (bDNA) (Figure A6 in the Appendix). Multiple, specific, synthetic oligonucleotides hybridize to the target and capture the target onto the surface of a microwell plate. Synthetic bDNA amplifier molecules and multiple copies of an alkaline phosphatase-linked probe are hybridized to the immobilized complex. Detection is achieved by incubating the complex with a chemiluminescent substrate and measuring the light emission. Since the target is not amplified, the signal is approximately proportional to the level of target nucleic acid. The quantity of specific target in the sample is determined from a standard curve.

### 11.1.4 Other Methods

The basic principles in these guidelines should be applied not only to the previously mentioned procedures, but also to new methods as they are developed and implemented. A number of new methods under development may make amplification unnecessary. One example is Invader®, which is particularly useful for detection of point mutations, such as those existing in the genes involved in hypercoagulation. In this system, two probes are designed for the target of interest such that there is a one-base overlap in the probes at the potentially mutated nucleotide. The probes compete with one another to hybridize at that site, and the resultant trinucleotide structure will be cleaved by the specific enzyme used in the reaction. If the nucleotide target has been altered, there will be no annealing of the probes, no trinucleotide structure, and no cleavage. The cleavage of the probe releases a nucleic acid "flap," which fuels a second reaction (all taking place in a single tube). This "flap," serves as a probe in the second round of competition/cleavage, this time releasing a fluorochrome that can be detected in the reaction mixture. The reaction takes place isothermally in a single tube, and results are available in a few hours.

## 11.2 Detection Systems

The sensitivity of current detection systems (in order of decreasing sensitivity) could be ranked as follows:

(1) capillary electrophoresis;

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(2) solid phase (blots, *in situ* hybridization, wells, beads);

(3) solution hybridization (size exclusion or hydroxyapatite, chromatography, affinity capture, homogenous);

(4) HPLC (PCR only); and

(5) gel electrophoresis.

However, the sensitivities will vary according to the type of probe (i.e., cloned or oligonucleotide); detection method (e.g., radioactive, fluorescent, chemiluminescent, enzyme, or hapten); and the hybridization format (e.g., sandwich capture or direct). Consequently, when any of these detection formats are used to detect amplified nucleic acid, the overall sensitivity will be much greater than direct detection alone.

### 11.2.1 Gel and Capillary Electrophoresis

The standard and most commonly used method for DNA (or RNA) detection is gel electrophoresis. The test principle is that DNA is negatively charged and will migrate towards the anode at neutral pH. The rates of migration of the molecules depend on their relative molecular masses and on the porosity of the gel. Large molecules migrate more slowly than small molecules. Therefore, a clear separation of DNA by size will be evident on the gel. The gel matrix can be either agarose or polyacrylamide gel (PAG) in a predetermined gel composition (%) dictated by the size of the nucleic acid being electrophoresed. For example, a 2% (w/v) agarose gel will efficiently separate 100- to 2,000-base DNA molecules, but it might not easily resolve larger fragments. Note that the physical form of the DNA (i.e., single-stranded or double-stranded linear, nicked circular, or superhelical circular) will cause it to migrate differently in different gels. Nucleic acid migration is affected by agarose or acrylamide concentration, current, ionic strength (i.e., buffer), and temperature. Finally, the addition of the fluorescent dye, ethidium bromide (WARNING: This substance is mutagenic) will enable visualization of the DNA under ultraviolet (UV) light. The detection limit of gel electrophoresis is ≈2 ng DNA. This format is rapid and simple to use in detecting DNA product from amplification reactions. If the electrophoresed product is Southern-blotted and hybridized with a labeled probe, the sensitivity improves to the range of 0.2 pg.

Capillary electrophoresis used as a molecular analytical tool holds promise for the greatest sensitivity of the detection formats listed. It is also amenable to automation. The principle of separation relies upon a charge-to-mass concept. The sample is introduced into a gel matrix (e.g., agarose or PAG), placed in buffer, and exposed to an electric field. Sample monitoring is performed by UV or some alternative detection system (e.g., fluorescence).

### 11.2.2 Solid Phase (Blots, *In Situ* Hybridization, Wells, Beads)

Southern blot analysis is the transfer (i.e., capillary, vacuum, or electrophoretic) of DNA to nitrocellulose or nylon support followed by probe hybridization. (**NOTE:** Northern blotting is the transfer and hybridization of RNA.) (See Figure 1.)

Direct blots use membranes for sample application and do not require a gel electrophoresis step. There are four types of direct blots: slot, dot, spot, and reverse dot. The slot and dot blots are basically the same except for the shape of the area containing the DNA. Both methods use vacuum to apply DNA to a defined area on either a nitrocellulose or nylon membrane, which is subsequently exposed to the probe during a hybridization reaction. The spot blot is the direct application of DNA to a membrane without vacuum. All three of these formats use a labeled probe to hybridize a solid phase-bound DNA target. In contrast, the reverse dot blot uses a membrane-bound probe (i.e., typically a 200- to 300-base poly-T-tailed probe is UV-cross-linked to the membrane) that specifically hybridizes to a target in solution. The

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reverse dot blot is more sensitive than the other three blots. An advantage of the solid-phase blot format is that it saves time. A disadvantage is the possibility of obtaining false-positive results. The reason is that direct blots, unlike the Southern blots, do not discern the size of the hybridizing DNA product as a check on specificity. Therefore, a higher stringency has to be used to ensure that the signal generated from the blot is specific for the target of interest.

The microtiter plate well and bead solid-phase formats offer good sensitivity and are well suited for automation. This format consists of binding the probe to a well/bead, followed by target capture. With the addition of another specific signal probe to react with the captured target nucleic acid, the specificity of this "sandwich" type nucleic acid hybridization is greatly enhanced. Many variations of this basic format exist, including the use of chemiluminescent detection, which is comparable or superior in sensitivity to the radioactivity-based systems.

*In situ* hybridization is a technique that uses intact cells containing specific DNA or RNA targets as substrates for hybridization with specifically labeled nucleic acid probes (i.e., radiolabeled, enzyme, or hapten). For example, detection of the Epstein-Barr virus (EBV) in formalin-embedded, paraffin sections uses this type of format. Disadvantages are that the technique is time consuming and requires "technical expertise." However, the rare EBV-infected cell can be detected with a sensitivity of 20 to 100 copies/cell. Coupled with PCR or signal amplification strategies, this format could prove to be an extremely sensitive diagnostic tool (i.e., 1 copy/cell sensitivity).

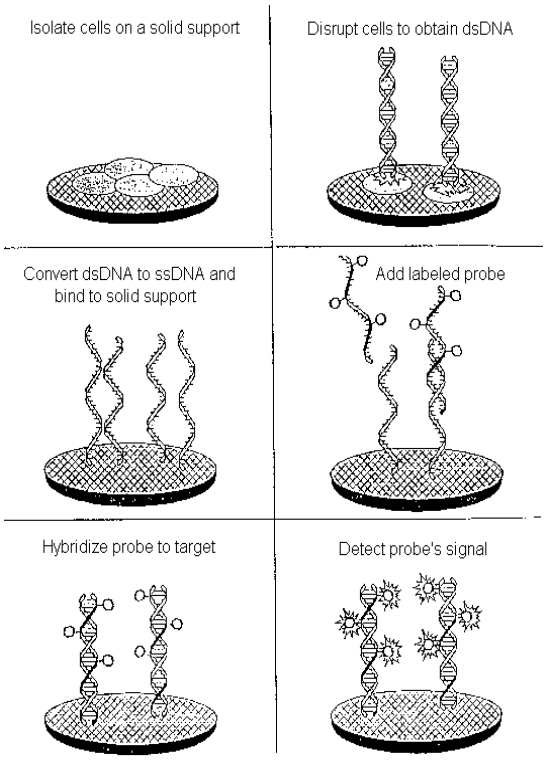


**Figure 1. Basic Steps in a DNA Probe Hybridization Assay.** From Wolcott MJ. Advances in nucleic acid-based detection methods. *Clin Microbiol Rev.* 1992;5:370-386. Reprinted with permission from American Society for Microbiology and Dr. MJ Wolcott.

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### 11.2.3 Solution Hybridization (Exclusion or Hydroxyapatite, Chromatography, Affinity Capture, Homogenous)

This method consists of hybridizing the probe to the target in a liquid phase, followed by either the removal of the unbound, labeled probe from the hybridized probe-target complex (e.g., exclusion chromatography) or capture of the hybrid (e.g., hydroxyapatite, charged magnetic particle or antibody). Separation can also be accomplished by gel electrophoresis. The signal from the labeled probe-target complex is then measured accordingly (e.g., $^{125}$I-probe gamma counter activity). Homogenous solution hybridization is different in that all steps occur in solution. Hybridization is accomplished by the use of two probes with different labels. A positive reaction occurs only if the two probes bind the target, wherein a signal is emitted due to probe interaction. Another variation of the homogenous assay is the hybrid protection assay where the hybrid protects a chemiluminescent-labeled probe from chemical hydrolysis.

Amplicons can be detected in a fairly easy, rapid, and sensitive manner using $^{32}$P-labeled oligoprobes. Thirty to fifty bp oligonucleotide probes are chosen and synthesized to be specific for the amplicon of interest. Radioactive end-labeling of the probe is accomplished enzymatically using polynucleotide kinase or terminal deoxynucleotidyl transferase (TdT). After annealing of the probe in solution to the postamplification reaction mixture, the sample is electrophoresed through an acrylamide minigel, then wrapped in plastic and used to generate an autoradiogram. Presence of a band appropriate in size for the amplicon indicates presence of the target, such as a chromosomal translocation. Presence of only the smaller probe band indicates absence of the target.

### 11.2.4 High Performance Liquid Chromatography (HPLC) (PCR only)

The advent of new anion-exchange nonporous columns offers a means of separating, quantifying, and identifying PCR products and PCR restriction fragments. This can usually be accomplished in less than 30 minutes. One disadvantage is the high cost of the HPLC instrument. Another disadvantage is that samples must be run sequentially and cannot be batch processed.

### 11.2.5 DNA Sequencing

DNA sequencing has traditionally been a research tool. As more genes are discovered and their sequences uncovered, sequencing is emerging as a powerful diagnostic tool as well.

#### 11.2.5.1 DNA Sequencing Using Radioactive Labels

In the mid-1970s, a method of DNA sequence analysis based on chemical degradation of DNA was described. An enzymatic method based on random chain termination with dideoxynucleotides was reported. The original development of these DNA sequence analysis methods was done using radioactive markers such as $^{32}$P or $^{35}$S. For the Sanger method, a reaction mix containing a primer, DNA polymerase, and the four deoxynucleotide triphosphates (at least one of which is radioactively labeled) is prepared and then divided into four aliquots. A limiting amount of either A, T, C, or G dideoxynucleotide triphosphate is added to each of the four aliquots so that the dideoxy-A tube ends up containing all of the fragments that contain terminal A, the dideoxy-T tube ends up containing all of the fragments that contain terminal T, and so on. After the sequencing reactions are completed, the A, T, C, and G aliquots are each loaded onto a separate lane of a denaturing acrylamide gel and separated by high-voltage electrophoresis. The gel is then subjected to autoradiography. The sequence is read by following the bands between the A, T, C, and G lanes. Accurate interpretation of base sequence from the autoradiograms requires skill and careful attention to detail. Computing densitometers, which can be used to measure the intensity of the bands from the exposed x-ray film, can also be used to facilitate interpretation of this data. However, variation in band mobility between lanes, and lanes that do not run straight down the gel, make radioactive sequencing difficult to interpret electronically. Chemistry anomalies, such as compressions and stops, also make instrument interpretation difficult. Several companies have written highly sophisticated software

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that interacts with a densitometric film scanner to try to address these problems. Traditional autoradiography can also be replaced with direct reading of the radioactive blot. Several instruments provide real-time visualization of the radioactive areas of the blot, and computer analysis of the counts coming from each area. Their software can also find lanes, detect bands, correct for "smiling," and read out the sequence. These systems are simple and easy to use. Their one drawback is that, if samples are weakly labeled, the instrument may be tied up for many hours counting a single blot.

This problem is addressed by phosphorimagers. With these instruments, the gel is then placed in a cassette that contains a medium to capture all the energy released by beta particles. The beta particles excite the medium which in turn emits a photon as it returns to the ground state. These photons are counted by the imagers and their locations determined. This medium has a greater dynamic range than conventional x-ray film and requires much shorter exposure times. Since the instrument is used only for reading the medium, it is possible to simultaneously expose multiple gels on multiple phosphor plates in a manner analogous to autoradiography.

11.2.5.2 DNA Sequencing Using Fluorescent Labels

Fluorescent DNA sequencing uses Sanger sequencing reactions run exactly as they are for radioactive sequencing, except that a fluorescently labeled primer or dideoxy terminator is substituted for the radioactive label. Dye-labeled primers for most of the common sequencing vectors, as well as dye-labeled dideoxy terminators, are commercially available. Custom dye-labeled primers can be synthesized easily. Several companies market fluorescent DNA sequencers which perform both the electrophoretic separation as well as the detection of the fluorescent bands. During electrophoresis, each sequencing fragment's fluorescence is excited by directing a laser through a small area in the glass plates near the bottom of the gel. Light emitted from the dye-labeled DNA fragments passing through this area is detected, and digitized signals are then transferred to a computer for interpretation. Software is used to analyze emission wavelength, peak height, shape, and interval, and to provide automatic base calling.

## 11.3 Selection and Qualification of Sequences

Following is a discussion of considerations involved in selection and qualification of nucleic acid sequences to be used as hybridization probes and primers. There are several options with respect to the choice of sequences comprising the probe, the length of the probe, the type of probe used (DNA or RNA), the source of the probe (synthetic, clone, genomic), and its purity. Each of these parameters, along with the stringency of the hybridization and wash conditions, affect the sensitivity and specificity of the hybridization reaction.

### 11.3.1 Hybridization Stringency

Nucleic acid hybridization reactions contain probe, target, salt, chaotropes, and buffer. They may contain blocking agents (Denhardt's solution, excess unlabeled DNA or RNA); an organic solvent (formamide); and an accelerator (dextran sulfate or polyethylene glycol). The concentration of salt, the presence and concentration of organic solvent, and the temperature at which the reaction occurs define the stringency of the reaction. At high stringency (conditions may include low salt and high formamide concentration, high temperature), only nucleic acid strands with a high degree of sequence complementarity will hybridize and remain hybridized. At low stringency (conditions may include high salt concentration, no formamide, low temperature), cross-hybridization and mismatches may be tolerated between DNA strands with imperfect homology.

### 11.3.2 Hybridization Probe Sequence Selection

The first step in probe development is to identify a unique and specific DNA or RNA sequence in the target of interest. Target sequences must be carefully chosen to minimize cross-hybridization. The G+C

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content of the target sequence (if known) should be selected to allow both favorable hybrid stability (melting temperature [Tm]) and discrimination between related sequences at the stringency of hybridization and chosen wash conditions. In addition, the success of some labeling protocols may be affected by the nucleotide composition of the probe. The nucleotide sequence contained within the probe may be only a small fraction of the target; the length of the probe also affects the melting temperature.

If the nucleotide sequence of the target is known, the Tm of a DNA-DNA probe-target hybrid can be estimated mathematically, e.g., using the following formula:

$$Tm = 81.5\ ^{o}C + 16.6(\log_{10}[Na^{+}]) + 0.41\ (\%\ G{+}C) - 0.63\ (\%\ HCHNO) - (600)/\ l$$

where $l$ = length of hybrid in base pairs.

RNA-RNA and RNA-DNA hybrids vary slightly from this formula. The melting temperature of a DNA-DNA or DNA-RNA hybrid will be substantially different in chaotropic salts. For oligonucleotides with less than 18 nucleotides, the Tm in degrees Celsius can be estimated by multiplying the number of A+T residues by two and the number of G+C residues by four, and adding the two numbers. The estimated melting temperature can be used to set initial parameters for hybridization and wash stringency.

When the nucleotide sequence of the target is known, computer programs can help in the selection of a probe. If the nucleotide sequence of the target is not known, or if the target population is heterogeneous, candidate probes must be empirically evaluated.

### 11.3.3 PCR Primer Sequence Selection

For selection of primers for PCR, the sequence of the target must be known. Although selection of successful primers may be somewhat empirical, there are some guidelines:

- Use a G+C content equal to that found in the amplified product (close to 50%).
- Avoid secondary structure in the primer, especially at the 3' end.
- Use primers that are noncomplementary to each other.
- Use primers between 20 and 30 bases long (or longer).

However, these guidelines do not guarantee success. DNA sequence analysis computer programs can also help in PCR primer selection.

### 11.3.4 Sensitivity and Specificity Challenge

After potential probes and/or primers are selected, they must be tested for sensitivity using appropriate positive material. In addition, specificity must be evaluated by testing for cross-hybridization with competing genetic material; this must be present in the test sample at a clinically relevant concentration. Whole cells should be used, rather than purified nucleic acid, to reflect any contribution made by cellular DNA and RNA and any effect of the sample preparation method. Finally, a challenge with human DNA (as found in white blood cells, epithelial cells, and tissue culture cells) should be included.

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### 11.3.5 Probe and Primer Forms and Purity

Nucleic acid sequences can be used in the following different forms for hybridization:

- synthetic oligonucleotides;
- cloned DNA;
- reverse-transcribed cDNA; and
- RNA.

The choice between these forms is influenced by the extent of knowledge about the target, specificity and sensitivity requirements for the assay, cost, purity, and available technology. In cases where the nucleotide sequence of the target is known, and where high specificity is required, synthetic oligonucleotides may be preferred.

To use oligonucleotides as primers for PCR, some purification may be necessary after synthesis. One option is to precipitate the oligonucleotide directly from the resuspended lyophilized material by addition of NaCl and ethanol. However, some loss of amplification efficiency may occur. The methods of choice are HPLC or, alternatively, gel purification, because it ensures that only full-length primers are present in subsequent reactions.

In occasional instances, genomic DNA, or fragments of genomic DNA cloned into a plasmid vector, may be used as a probe. In contrast to short, synthetic oligonucleotides, larger DNA (or RNA) molecules can be labeled to high specific activity to increase test sensitivity, can be inexpensive to obtain, and can help to overcome population heterogeneity problems. However, if the specific activity is too high, e.g., greater than $2 \times 10^8$ dpm/µg, probe specificity can be reduced by radiolytic damage to bases adjacent to the labeled bases. Also, these materials must be purified from growing cells and contaminating substances. Use of whole genomic DNA as a probe may cause reproducibility problems. If genomic DNA is used as a probe, or if the plasmid vector is included in the probe, then adequate controls must be included in the assay to demonstrate that specificity is not compromised.

## 11.4 Controlling False-Positive Nucleic Acid Target Amplification Reactions

A significant challenge facing the diagnostic application of nucleic acid amplification methods is the occurrence of false-positive results due to contaminating nucleic acids. The ability of amplification techniques to produce large numbers of copies of a sequence from minute quantities of nucleic acid necessitates that extreme care be taken to avoid false-positive results due to transfer of DNA between samples.

Nucleic acid contamination stems from three major sources:

- In the clinical laboratory setting, it may be the result of specimens containing actual target molecules or cells from another sample.
- In the development of an amplification technique, it may be the result of contamination of amplification reagents with plasmid or phage DNA.
- It may be the result of accumulation of amplified product that can result in the contamination of reagents, buffers, laboratory glassware, autoclaves, and ventilation systems.

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Specific laboratory practices and procedures should be implemented to minimize the occurrence of false-positive results. Use of chemical inactivation techniques may also be employed to prevent reamplification of any contaminating amplicons. Chemical inactivation does not, however, prevent false-positive results due to carry-over from other samples or cloned targets used in the laboratory.

### 11.4.1 Reagents and Solutions

All reagents used in nucleic acid amplification should be prepared, divided into aliquots, and stored in an area that is separate from the specimen preparation or postamplification area. Dedicated equipment and supplies should be used. Oligonucleotides should be synthesized and purified in a clean, amplification-product-free environment. Once reaction conditions have been optimized, reagents can be premixed into master mixes. These master mixes can be divided into aliquots of the volumes required for each reaction run. This will minimize the number of samplings and reduce the potential for contamination. The reagent lot number should be recorded so that if carry-over does occur, the source can be easily identified.

### 11.4.2 Pipettors

Separate pipettors should be used for reagent preparation, specimen preparation, and postamplification analysis. Pipettors used for nucleic acid amplification set-up should always be separated from amplified products and should remain in the area in which they are used. Positive displacement pipettes or plugged pipette tips should be used to prevent contamination of pipettor barrels by aerosols. All pipettors should be cleaned at regular intervals with 5 to 10% freshly prepared bleach.

### 11.4.3 Laboratory Practices

#### 11.4.3.1 Workflow

Each laboratory should develop its own unidirectional workflow from “clean” to “dirty” areas. The use of a unidirectional workflow will reduce the opportunity for contamination to occur. Color-coded equipment, reagents, and supplies may help ensure that a unidirectional workflow is maintained.

#### 11.4.3.2 Laboratory Coats

If feasible, laboratory coats should be dedicated to areas and changed when traveling between the reagent preparation area, sample preparation area, and amplification and detection areas.

#### 11.4.3.3 Gloves

To reduce the possibility of introducing amplified nucleic acid into the clean areas, disposable gloves should be worn and changed when entering or reentering the amplification preparation area. Disposable gloves may be changed between samples to prevent cross-contamination between samples.

#### 11.4.3.4 Uncapping Reaction Tubes

To force any liquid down from the sides, it is recommended that tubes be subjected to a quick centrifugation before uncapping. Tubes should be uncapped carefully to prevent aerosolization.

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11.4.3.5 Addition of Reaction Components

Nonsample components (mineral oil, dNTPs, primers, buffer, and enzymes) should be added to the amplification reactions before the addition of the sample. Before proceeding to the next tube, each tube should be capped after the addition of the sample.

### 11.4.4 Selection and Preparation of Controls

11.4.4.1 Positive Controls

A positive control that amplifies weakly but consistently should be selected. The use of dilute positive controls prevents the unnecessary generation of large amounts of amplified product that can result in contamination. This positive control material must be appropriately characterized (i.e., concentration, template copy number, sequence, source).

11.4.4.2 Reagent Blanks

Applicable reagent controls should be included with each amplification batch run. These controls contain all of the necessary components of the reaction without the addition of template nucleic acid or human DNA.

11.4.4.3 Negative Controls

Negative controls should be dispensed last so that they reflect the state of the reagents added.

### 11.4.5 Laboratory Design

Ideally, three physically separate areas of the laboratory should be available for reagent preparation, specimen preparation, and amplification and product detection. The reagent preparation area, for those laboratories using only commercially available kits, is considered to be at the site of manufacture. In laboratories where enzymatic or chemical means of inactivating amplified products are used, the demands for physical separation of pre- and postamplification procedures may be somewhat reduced, but good laboratory practice should still be diligently exercised.

11.4.5.1 Workflow

Specimens should be processed in an area of the laboratory that is isolated from amplification and detection areas. Ideally, the specimen preparation area should be under positive pressure to other areas of the laboratory. If the specimen preparation area cannot be maintained at a positive pressure to other areas of the laboratory, specimen preparation should be performed in a Class II biological safety cabinet to prevent contamination. The pre- and postamplification laboratories should be served by separate ventilation systems. Also, the postamplification area should be under negative pressure. Personnel traffic should go from the specimen preparation area with change in laboratory coats before going to the pre- and postamplification areas. Laboratory coats should be dedicated to areas and changed when going in and out of each area. With the introduction of commercially licensed tests and new methods, some of these requirements may be reduced. However, if contamination becomes a problem, then separate areas of the laboratory should be devised to accommodate these different processing steps.

11.4.5.2 Containment Devices

In the event that separate laboratory space is not available to segregate pre- and postamplification activities, a Class II biological safety cabinet should be used as a containment device for specimen preparation. Class I safety cabinets do not provide protection for material contained within them. Dead air

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boxes with ultraviolet light attachments can provide a clean bench area for specimen preparation in a dedicated specimen preparation laboratory; UV lamps lose energy efficiency over time.

### 11.4.6 Amplification Product Inactivation Methods

Several amplification product inactivation methods have been described for use with PCR; many of these may be useful for inactivating the products of other amplification methods. Because PCR is currently the amplification method used most commonly, this section describes these techniques as they pertain to PCR and provides guidelines for their implementation in PCR protocols. Preamplification UV light irradiation for contamination control has also been described, but its reliability as an inactivation method has not been proven; thus, it is not discussed here.

#### 11.4.6.1 Enzymatic Inactivation

In the enzymatic method, deoxyuridine 5' triphosphate (dUTP) is substituted for deoxythymidine 5' triphosphate (dTTP) in all amplification reactions, which results in incorporation of U in place of T in the amplification product. Thus, amplification products can be distinguished from authentic target DNA by the presence of an "unnatural" nucleotide base. The bacterial enzyme uracil-N-glycoylase (UNG) is then added to the reaction mixes. During a brief incubation step before amplification, uracil-containing DNA strands that are carried over from previous amplifications are enzymatically degraded and thus cannot serve as substrates for further amplification. Then, the UNG is inactivated by heating it to 94 ºC. Because naturally occurring target DNA does not contain large numbers of uracil residues, this method distinguishes between amplification products carried over from previous reactions and the DNA from an organism in a clinical specimen. Thus, the UNG protocol allows active templates to be accumulated in the laboratory, but a "pre-PCR" inactivation step selectively eliminates them before the amplification of a new sample set.

**NOTE:** UNG may be reactivated upon incubation at an ambient temperature following amplification and begin to digest newly amplified products. A method of UNG inactivation such as chemical denaturation should be employed soon after the amplification procedure to prevent the digestion of the newly amplified products. Alternatively, thermolabile UNG can be heat-inactivated.

**CAUTION:** Substitution of dUTP for dTTP will result in failure of restriction enzymes to recognize sites that include a T residue, thus causing assay failure for PCR-RFLP assays that depend upon recognition of a thymidine-containing restriction site.

#### 11.4.6.2 Optimization of Amplification Reactions

As for all of the inactivation methods, amplification reactions incorporating UNG inactivation should be carefully optimized. Primers should be chosen so that amplification products are large enough to be inactivated efficiently, yet not so large as to reduce amplification efficiency. The efficiency of the UNG protocol depends, to some degree, on the concentration of target residues in the product. G+C-rich products may be more difficult to inactivate due to the lower concentration of uracil residues. Some investigators have observed a loss of sensitivity after incorporation of the UNG protocol. However, this is not likely to be a universal experience, considering the advantages of the "enzymatic hot start" that is a feature of UNG inactivation.

##### 11.4.6.2.1 dUTP Concentration

The optimal dUTP concentration that will allow efficient amplification should be determined empirically. Many protocols will tolerate complete substitution of dUTP, but some will require either an increase in dUTP concentration relative to the other dNTPs or a mixture of dTTP and dUTP. This may depend, to some degree, on the G+C content of the target sequence.

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11.4.6.2.2 UNG Reactivation

UNG will regain activity, even after multiple cycles of amplification, if temperatures within the reaction mixture drop below 55 °C. Residual UNG activity may affect reaction sensitivity if the products are held at room temperature before gel loading or slot blotting. Thus, reaction components should be held at 72°C, or they should be chemically treated with NaOH, before analysis. This will prevent postamplification degradation and the resultant loss of signal. In addition, primers and protocols should be designed so that primer-annealing temperatures greater than 55 °C are employed in thermal cycling profiles. Thermolabile UNG is available and capable of being inactivated more readily.

11.4.6.2.3 Probe Hybridization

dUTP substitution may reduce the affinity of internal oligonucleotide probes that are used to confirm the presence of specific PCR products. In general, the reduced affinity can be overcome by reducing the stringency of probe hybridization or washing.

11.4.6.3 Photochemical Inactivation

An alternative "post-PCR" method has been described that exploits the photochemical properties of soluble psoralen derivatives. These compounds are added to the PCR reaction mixture before amplification; they do not substantially interfere with primer annealing or *Taq* polymerase activity, and they are thermally stable. After amplification (but before the reaction vessels are opened), the tubes are exposed to long-wave UV light, which penetrates the polypropylene tubes and photochemically activates the psoralen derivative but does not otherwise damage the DNA. The activated psoralen then forms cyclobutane adducts with pyrimidine residues on the amplified DNA that prevent *Taq* polymerase from traversing the molecule in a subsequent amplification. The efficiency of this process depends on the length and nucleotide base composition of the amplified product. In general, for amplified products greater than 300 base pairs in length with roughly 50% G+C content, near-complete inactivation can be achieved. Also, unlike the enzymatic methods, the original target DNA is inactivated in addition to the amplified products. While this results in reduced risk of target accumulation from treated reactions, it also prevents subsequent amplifications from targeting other sequences present in the reactions. The conditions and requirements for postamplification photochemical inactivation of PCR-amplified products were recently described.

The following parameters should be considered when using photochemical inactivation:

- Amplified Product. The fraction of strands that escapes the photochemical modification, and is therefore not inactivated, is determined by the length and sequence of amplified product and the concentration of the sterilization reagent. Since A+T-rich sequences are more reactive with psoralens, the judicious choice of primers that generate long (greater than 200 to 300 base pairs, depending on G+C content) or A+T-rich products facilitates the inactivation process. Inactivation efficiency is also improved by increasing the isopsoralen concentration.

- Isopsoralen Concentration. The most useful range of concentrations is 25 to 200 µg/mL. Higher concentrations of the positively charged reagents compromise detection sensitivity by either inhibiting the amplification process or by reducing the hybridization efficiency of the inactivated products. Partial reduction of the inhibition of PCR amplification is achieved by the addition of up to 10% glycerol as a cosolvent in the amplification mixture. The number of PCR cycles should be adjusted to bring the detection level back to the level that exists in the absence of the inactivation conditions. Typically, two to three additional cycles of amplification are necessary for protocols using high concentrations of isopsoralen.

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- Product Mobility and Hybridization**.** The effects of isopsoralen concentration on the mobility and hybridization of an amplified target can be substantial. Often, there is a concentration-dependent shift in the apparent molecular weight of the product. At various isopsoralen concentrations without UV exposure, there may also be a slight inhibitory effect on amplification efficiency at the highest isopsoralen concentration. In addition, there may also be a small concentration-dependent decrease in hybridization efficiency that is most pronounced at the higher isopsoralen concentrations.

  Amplification efficiency in the presence of isopsoralen should be evaluated under conditions that are far removed from the PCR plateau so that inhibitory effects are visible. Using tolerable concentrations of isopsoralen, the hybridization efficiency of the modified amplified products should then be determined.

#### 11.4.6.4 Chemical Inactivation Protocols

An amplified product inactivation method based on selective base hydrolysis of ribose linkages is available. The effectiveness of primer hydrolysis as an inactivation protocol has not been fully evaluated at this time; however, the capacity of the method to inactivate amplified PCR products is approximately $10^6$ molecules.

##### 11.4.6.4.1 Hydroxylamine Treatment

Another postamplification inactivation method relies on the ability of hydroxylamine to produce covalent modifications of DNA. Hydroxylamine reacts preferentially with the oxygen atoms in cytosine residues, which creates covalent adducts that prevent base pairing with guanine residues. The presumed mode of action of hydroxylamine for inactivation of PCR products is to modify primer binding sites and intervening template sequences so that annealing and reamplification cannot occur. However, the use of hydroxylamine currently requires opening the reaction tubes or injecting the compound into tubes using a syringe, which exposes the reaction components to the outside. Thus, the usefulness of this approach as a long-term contamination control strategy is unknown at present.

##### 11.4.6.4.2 Topical Inactivation Methods

Topical inactivation methods exist that can be used to clean up spills of reaction mixtures, as well as for cleaning work surfaces as part of routine QC programs. The most useful and effective method to date is the application of bleach (approximately 5% sodium hypochlorite prepared fresh daily). Ultraviolet lights placed over working surfaces may also be effective in controlling contamination. For maximum effectiveness, the UV source should be within two feet of the working surface in order to provide a dosage adequate to cross-link small DNA targets.

**NOTE:** UV light is only marginally active when irradiation is limited to one or two hours; overnight irradiation may be necessary for eliminating amplification products of 200 base pairs or larger.

#### 11.4.6.5 Validation of an Amplified Product Inactivation Method

Once amplification reactions are optimized with inactivation protocols in place, an assessment should be made of the effectiveness of the contamination control measures by intentionally contaminating reaction mixtures with dilutions of modified product and comparing the results with untreated controls.

Later, if a laboratory decides to switch from one inactivation method to another, it should validate the new method, as previously stated, before implementing the change.

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## 11.5 Test Validation

**NOTE 1:** This section addresses issues to be considered by laboratories developing molecular-based diagnostic tests: either industry or clinical laboratories. Laboratories that use diagnostic test kits according to manufacturers' instructions should adopt the QA recommendations presented in the next section. However, if a laboratory is developing its own method or altering an approved commercial method, the following issues (as applicable to the situation) should be considered in the validation of the new test or test alteration.

**NOTE 2:** These recommendations provide useful information on definitions and clinical validations of molecular diagnostic methods, sources of control material, and statistical testing.[b,c,d] The recommendations complement the guidelines found in this and other NCCLS documents.

**NOTE 3:** To address issues basic to most common molecular methods, considerations for each component part, as well as a complete system, are described. Laboratorians should implement those sections appropriate to their test methodologies (i.e., if a test system incorporates DNA hybridization only, the amplification issues should be disregarded).

Test validation should be conducted before a new test is introduced for use. The test should be subjected to:

- thorough literature review, and
- analytic and laboratory/clinical correlation studies to:
  - characterize the locus/mutation(s) being detected;
  - establish the performance properties of the test to ensure the test's ability to provide consistent and reliable results;
  - establish the clinical utility of the test (i.e., that the test will measurably contribute to the diagnosis of a disease);
  - define aspects of the procedure which should be carefully regulated to maintain test performance; and
  - define relevant limitations of the test.

Such validation is necessary to assure the safe and effective use of a genetic test for its intended use. Detailed protocols for test validation are beyond the scope of this document. This section will provide guidance on key points to consider for validation purposes. Each laboratory should develop its own validation protocols.

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[b] Guidance on validation of new *in vitro* diagnostic products can be obtained from the Appendix in the FDA's *Medical Device Good Manufacturing Practices Manual.* 5th ed. DHHS; 1991.

[c] Laboratories are directed to review the CLIA '88 requirements for quality assurance published in the *Federal Register*, (February 28, 1992).

[d] The Association for Molecular Pathology has published a detailed set of recommendations for in-house development of molecular diagnostic tests (*Am J Clin Path*. 1999;111:449-463).

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### 11.5.1 Characterize the Target Locus/Allele/Mutation Being Detected by the Test

In general, an association between a disease state and a particular marker should be published and confirmed by a minimum of three independent investigators prior to being considered for diagnostic use. The utility from the clinical standpoint should be documented.

The rationale for selecting the target of interest should be fully justified. Each target locus/allele/mutation being detected by the test should be fully characterized and well documented in the scientific literature. Characterization should include the following, as applicable:

- map position of target locus—recorded in International Human Gene Mapping Workshop, Geneatlas, GenBank, Genome Data Base (GDB), or the International System for Human Cytogenetic Nomenclature (ISCN), etc.;

- type of defect, number and type of allelic mutations, target sequence being detected, etc.;

- polymorphic nature of the target and the degree of conservation, if relevant;

- target population for whom the test is intended;

- population distribution of the disorder, frequency of the defect, mutation rates, and any known variability in the frequency distribution between subpopulations (e.g., geographic, racial, ethnic, etc.); and

- genetic basis and mechanisms relevant for expression of the clinical disorder, including inheritance pattern as validated by family studies; genetic heterogeneity; reduced penetrance; variable expressivity; delayed onset; and any other unusual phenomenon responsible for expression of the disorder (e.g., uniparental disomy, imprinting, microsatellite/triplet expansion, mosaicism, mitochondrial basis, etc.).

#### 11.5.1.1 Method/Procedure Validation

The following steps outline procedure/method validation:

(1) Identify the critical aspects of a procedure that should be carefully controlled and monitored to provide consistent and reliable results.

(2) Substantiate application of the methodology to detect the target in question, including appropriate peer-reviewed literature references.

(3) Establish the minimum amount/quantity of tissue/specimen needed to obtain reliable results.

(4) Validate each step of the analytic procedure, QC, equipment, and instrument.

(5) Assure characterization of all critical reagents (e.g., enzymes, probes, primer, etc.) utilized by the procedure. Consider the following properties, as applicable:

   - Size; type of primer/probe (DNA/RNA); restriction enzyme map; base sequence; recognition site; probe label, manner and stability of attachment; function of multiple probes/primers; etc.

   - Sequence accession number if registered with data bank; licensure/patent information.

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- Rationale for reagent (e.g., probe/primer) design.

- Reagent specifications, manufacturing, and QC. For example, probe production: Cloning vector, vector size, percent insert, isolation, purity, conjugation/attachment, optimal concentration, functional validation.

- Acceptance/rejection criteria.

#### 11.5.1.2 Amplification

For tests employing an amplification procedure before detection, the amplification method must be validated separately first, then in conjunction with the whole system. Included in the validation are the performance of QA procedures on each of the key components of the amplification process and then functional testing of the amplification itself (i.e., sensitivity and specificity). (Table 5 in Section 11.6 summarizes the following QA strategies.)

#### 11.5.1.3 Oligonucleotide Primers

When purchasing oligonucleotides to be used in amplification methods, laboratories should select vendors that will supply a certificate of analysis with each shipment. The certificate of analysis should state the sequence (including identification and location of any attachment molecules); the molecular weight of the oligonucleotide without the attachments; the purity by an appropriate method (e.g., HPLC, capillary electrophoresis, and/or polyacrylamide gel electrophoresis [PAGE]); and base composition analysis. The certificate of analysis for each lot should be examined for the specifications established by the laboratory, and it should include stability and storage conditions. For example, for purity, expected results will vary with systems; however, vendors have been able to attain oligonucleotide with 95% purity. Only a single band of the "correct" size is acceptable for PAGE. The base composition analysis will vary with each sequence, and the percentages provided by the manufacturer's analysis should conform to those predicted by the sequence.

Laboratories synthesizing their own oligonucleotides should purify them by HPLC or another appropriate method, and the tests noted above should be part of the release criteria for any lot used in diagnostic testing. Each new lot of oligonucleotide synthesized or purchased is tested to confirm concentration, purity, and function.

##### 11.5.1.3.1 Purity

To assess purity, oligonucleotides can be electrophoresed on acrylamide gels. A single band should be observed.

##### 11.5.1.3.2 Attachments

Molecular attachments can be checked by analytical assays (i.e., biotin-avidin assay), incorporation of radioactivity, purity in separating methods (electrophoresis or chromatography), or by a functional comparison with a proven lot.

##### 11.5.1.3.3 Concentration

Several methods can be used to verify and adjust concentration. Choice of methods depends on the purity of the oligonucleotide being measured and the precision needed. One simple method is to make dilutions of the oligonucleotide, read the absorbance on a spectrophotometer at 260 nm using quartz glass cuvettes, and make calculations based on the molecular weight. Oligonucleotides can be diluted in water or buffer

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and the absorbance read at 260 nm. At 260 nm, one absorbance unit indicates 50 µg/mL of double-stranded DNA, approximately 37 µg/mL of single-stranded DNA, and 40 µg/mL of single-stranded RNA.

11.5.1.3.4 Functional Validation to Detect Contaminating Nucleic Acid Sequences

When oligonucleotides are used as primers in an amplification method, each new lot should be checked for contamination by being used in a significant but realistic number of negative reactions, e.g., five to ten replicates. No positive results should be observed.

11.5.1.3.5 Functional Validation to Determine Efficacy

Functional checks for primers and probes are accomplished in the same manner as crossing over serology kits or new lots of chemistry reagents: The old primers and probes are run with the new ones, in one run with the same samples. The samples should be a mixture of known controls and patient samples.

11.5.1.4 Polymerases and Other Nucleic Acid Modification Enzymes

Laboratories should select vendors that provide QA information with the enzymes. Information about the minimum activity characteristics and the conditions under which those characteristics are tested should be provided. When purchasing materials from vendors with QA programs, each new lot of enzymes needs only be tested functionally. Many laboratories accomplish this by making reagents with the enzymes and running the assay with the old lot versus the new lot using known controls and patient samples.

For methods employing RT-PCR, the reverse transcriptase (RT) and DNA polymerase activity of the enzyme(s) used should be evaluated. The RT activity may be assessed using DNAse-treated samples.

11.5.1.5 Other Components (Nucleotides, Buffers)

Depending on the importance of a reagent in the amplification reaction or its probability of being contaminated with extraneous nucleic acid sequences, purity and functional checks should be performed (i.e., amplifying a series of negative samples or reagent negatives with the new reagent). Buffers and deoxyribonucleoside triphosphates (dNTPs) should be subjected to functional tests when new lots arrive or are prepared, because alterations in their composition can adversely affect the reaction.

11.5.1.6 Amplification Controls

Controls employed to assess amplification include positive and negative controls and those used to assess the presence of inhibitors, such as the endogenous nucleic acid control. If amplification inhibitors of a particular target are suspected, duplicate samples can be run for each patient, and one sample can be spiked with a low level of the target in question (ideally near the lower limit of detection). When analyzing the results, negatives will be recorded only for those samples in which the spiked duplicate was amplified. As with sample preparation controls, a negative amplification control consisting of water or buffer in amplification reagents should be instituted at this point. The number of negative controls and their strategic placement will depend on the same conditions as described in Section 9.4 on sample preparation control.

11.5.1.7 Analytical Sensitivity and Specificity

Amplification-based tests must first be validated using purified target nucleic acid, cloned DNA, cell lines, or previously well-characterized clinical samples. When this phase is completed, the assay should be reassessed using multiple clinical samples, and appropriate adjustments made to optimize sensitivity and specificity. A sufficiently large number of clinical samples to reflect the level of molecular

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heterogeneity and nucleic acid degradation likely to be found in routine clinical practice should be employed.

Detection systems, particularly if they involve nucleic acid probes, should be evaluated independently before assessing their sensitivity and specificity as part of the complete system. Once the detection system has been optimized, the total system should be evaluated with clinical specimens and modified to optimize performance.

11.5.1.8 Detection (Nucleic Acid Hybridization) Probes

The performance of QA procedures on nucleic acid probes (oligonucleotide, double-stranded DNA, and RNA) is discussed in this section. Oligonucleotide and RNA probes may be synthesized by a laboratory or purchased from an established vendor. Double-stranded DNA probes may be either cloned DNA sequences of DNA fragments generated by the endonuclease digestion of genomic or plasmid DNA samples generated in the laboratory, or purchased from an established vendor. If double-stranded DNA probes contain any vector or nontarget sequences, the probes must be validated to prove that the vector or nontarget sequences do not compromise the specificity of the hybridization reaction. Negative controls containing such vector or nontarget sequences may be included in the assay. Other characteristics that should be tested include:

11.5.1.8.1 Probe Labels

Probes will have several types of reporter molecules or attachment molecules to link them to reporter molecules in assay systems. For all attachments (radioisotope labels, enzyme labels, other molecules), the labeling efficiency must be assured. In the case of radioisotopic, enzymatic, fluorescent, and chemiluminescent labels, the analytical range must be established and tested. For radioisotopes, establishing criteria for a minimum and maximum incorporation of label is important (i.e., specific activity in cpm/pmole). In validating a test system, the probe should be used with the label incorporated at the range of standard performance. The assay should be reproducible at the lower limits of detection.

11.5.1.8.2 Kinases

To check new lots of kinases, a functional test may be performed by labeling the new lot in parallel with an historical lot, using the same probe, or by determining the specific activity of the kinase.

11.5.1.9 Restriction Enzymes

Restriction enzymes used to characterize or identify diagnostic patterns should be checked with each new lot. Manufacturers provide information in their package insert material about the restriction pattern of specific plasmids. The restriction patterns should be duplicated in the laboratory before using the enzyme diagnostically.

11.5.1.10 Hybridization

In optimizing hybridization assays, the system should be run near the limits of hybridization stringency and still produce accurate results near the lowest detection limit. Laboratories should also run potentially cross-reacting organisms to confirm the specificity of the assay.

11.5.1.11 Equipment Validation

Before the introduction of "all-inclusive," automated clinical instruments for molecular microbiology, laboratories should run assays in semimanual formats. This requires careful assessment of the functional reliability of the instruments and equipment employed. Sample processors, incubators, thermal cyclers,

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water baths, heating blocks, mechanical plate washers, centrifuges, and pipettors are some of the instruments and equipment that may be used. Quality assurance, including validation, calibration, and record keeping on instruments and equipment is traditionally a required standard in clinical laboratories. Deciding which tests to perform to assess instrument functionality and the periodicity of the tests requires the individual judgment of each laboratory organization (see Table 7 in Section 11.6).

11.5.1.12 Common Instruments and Equipment

Most of the instruments and equipment used in molecular-based assays consist of pipettors, water baths, heating blocks, and detection-based equipment. Because small amounts of reagents and samples are used, pipettors should be calibrated at least twice a year. This can be done using the standard method: measuring the weight of water delivered by the pipette. Pipette manufacturers have different claims and warranties, which should be consulted.

Water baths and heating blocks should be checked with a calibrated thermometer each time they are used. In laboratories where assays are run continuously, temperatures are recorded daily. Where hybridization or other reactions are performed at room temperature, the daily room temperature is recorded. This is consistent with current good laboratory procedures in clinical laboratories.

Detection-based instruments may include spectrophotometers, enzyme immunoassay microtiter plate readers, fluorometers, luminometers, and fluorescent or light microscopes. These instruments should be calibrated and checked at least twice a year within the ranges used for molecular diagnostic testing.

11.5.1.13 Amplification Instruments for Technologies Requiring Thermal Cycling

Thermal cyclers are central to many amplification protocols and have been used for several years. As such, recommendations for maintaining them and assessing their reliability have been developed by instrument manufacturers. Minimally, the recommendations from manufacturers should be followed; otherwise, calibrate at least twice a year. Laboratories may choose to institute additional controls, especially while developing and validating assays. In addition to the internal diagnostic checks that may be built into the instrument, further assurances include monitoring reaction temperatures, printing out and checking cycles, and performing functional amplification checks.

To monitor the internal temperature of the wells of thermal cyclers, a type K thermocouple probe, minivolt converter, and chart recorder can be used to detect large variations (greater than 1 °C) between wells. The thermocouple should be placed in a reaction tube and inserted into the well of the thermal cycler during a typical run program. The thermocouple should be moved from well to well, testing at least six distant wells in a 48-well instrument. Though the probe can be moved during the program, allow at least one cycle for equilibration after moving it to a new well. Record the temperatures for all programmed steps. To determine functional specifications, consult the instrument manual for the claims.

Because temperature readings depend on the precision of the unit, a good thermocouple is necessary and must be calibrated against a temperature standard. Some instrument manufacturers sell temperature probes specifically designed to fit into the wells of their thermal cyclers. They are especially easy to use, and their calibration is guaranteed for a specified amount of time.

The frequency of this type of temperature monitoring depends on the type and history of the thermal cycler, and the amount of use. For reliable instruments used daily in diagnostic testing, temperature monitoring should be done monthly. For instruments used infrequently, temperature monitoring may be done less frequently; for less reliable instruments, more frequent temperature monitoring is recommended.

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In addition to the temperature check, another QA check is to print out the amplification programs with each run. (Some thermal cyclers have ports that allow the attachment of a printer for this purpose.) The printout will prove that the correct program was used for the assay, record that the proper number of cycles was used, and record any breaks in the cycling. By examining the times recorded to obtain each of the temperature plateaus, one may be able to observe instrument malfunction before it becomes apparent elsewhere. (Any trends or shifts in the time an instrument takes to achieve a programmed temperature should be considered warnings.)

A third type of test is the functional check which should be done when validating a new system or a new type of instrument. The functional check is accomplished by amplifying a low-level target in each of the wells. Deciding which tests to perform to assess instrument functionality and the periodicity of the tests requires the individual judgment of each laboratory organization (see Table 7 in Section 11.6).

### 11.5.2 Assess Performance Properties of the Test

The analytic and clinical/diagnostic performance properties of the test should be documented by studies that set quantitative goals for performance of the assay and consider important sources of variability using statistical procedures that quantitate their effects. Data are statistically sufficient if there are enough data gathered under the right conditions to give adequate confidence that the procedure will perform appropriately in the working laboratory setting for all intended uses/indications for use of the assay. Because of the adverse consequences of a missed diagnosis, goals should be set to achieve a standard of essentially zero error for analytic performance and accuracy in test interpretation.

Acceptable performance characteristics for a test will depend on the particular test applications, e.g., screening vs. diagnosis/confirmatory testing. Determination of acceptable performance for a test should be made on a case-by-case basis.

#### 11.5.2.1 Analytic Performance

Identify and characterize the critical analytic performance properties relevant to assuring consistent and reliable results. At a minimum, the analytic sensitivity, analytic specificity, and reproducibility of the assay should be evaluated.

The test should be validated for all specimen types (e.g., blood, bone marrow, etc.) that will be utilized for testing.

Evaluate the procedure using known, well-characterized specimens subjected to both recommended and extreme environmental shipping and storage conditions.

Analytic performance should first be characterized using known specimens or purified target DNA, as appropriate. When this phase is completed, the assay should be reassessed using clinical samples or control materials and appropriate adjustments made to optimize the procedure.

#### 11.5.2.2 Diagnostic Performance

Establish the diagnostic performance properties of the test for its intended use and specified target population(s) with appropriate studies. Molecular methods should be evaluated in comparison to a "gold standard" when a standard exists which is sufficient to diagnose a particular disorder. A new methodology should be implemented only if the performance characteristics are demonstrated to be as good as or better than existing methods or if the new methodology provides a significant new advantage, e.g., increased accessibility, improved turnaround time, justifiable cost benefit, etc.

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As is frequently the case in molecular diagnostics, a confirmatory "gold standard" is not available to use in comparison studies with molecular methods. Molecular methods are frequently superior to existing methods and may lead to the definition of previously unrecognized disease entities or to the definition of a clinical syndrome. In the absence of a "gold standard," identify the diagnostic endpoint that will permit a statistically measurable hypothesis. In instances where only appearance of the disease itself provides confirmation, clinical manifestation may be the desirable endpoint.

11.5.2.3 Establish Expected Values, Cut-Off Value(s), or Endpoint(s) for the Assay for Each Relevant Genetic State of Interest

When appropriate, establish the assay cut-off for distinguishing between pertinent states of nature. For example, for fragile X syndrome analysis, establish the size of triplet expansion that distinguishes between normal, permutation, and full mutations, using appropriate statistical methods. Evaluate specimens from well-defined/characterized individuals reflective of the target population(s). Document the diagnostic criteria for characterizing the subjects in the study population.

Determine the expected prevalence/frequency of the mutation in the relevant target population(s). The distribution and prevalence within the target population should be assessed using appropriate subject selection criteria. For example, determine the appropriateness of using specimens from unrelated vs. related individuals. Document any known diversity in expected prevalence between relevant subpopulations and other variables as appropriate.

11.5.2.4 Diagnostic Sensitivity and Specificity

Two key performance parameters, diagnostic sensitivity and specificity, are important for evaluating the test's safety and effectiveness. Well-designed methods comparison or clinical trials are crucial to establishing a test's diagnostic performance characteristics. Disorders with low prevalence, reduced penetrance, variable expressivity, delayed onset, genetic heterogeneity, and any other unusual phenomenon present unique study design challenges.

The diagnostic sensitivity and specificity of the test should be established using a sufficiently large number of subjects from the appropriate target population(s) so that predictive values together with their confidence limits can be adequately estimated for a range of population prevalences.

Effort should be made to avoid introducing biases when designing study protocols. Subject selection criteria (e.g., inclusion/exclusion criteria, randomization, etc.) should be carefully developed. The appropriateness of including related vs. unrelated individuals should be considered.

In cases of low-prevalence disorders or disorders with a long lag-time between testing and onset of disease, it may not be feasible to test the diagnostic performance of the tests in the intended use population. It has been recommended that in these special situations, a reasonable approximation of the performance of the tests may be determined using subjects with overt disease to test for sensitivity and nonaffected subjects beyond the age at which the disease usually becomes manifest to estimate specificity. Test interpretation should take into account any biases in estimates of these parameters introduced by less-than-ideal study design.

Validation of tests intended for prenatal diagnosis, in which fetal test results indicate the presence/absence of disease, will depend on follow-up of prenatal test results or on test results in living subjects.

Prospective studies (under the Institutional Review Board [IRB] and informed consent) to support the intended use/indications for use of the test are desirable, although studies performed with archived specimens may provide appropriate information. However, the investigator should be cognizant of the legal/ethical issues that may arise from use of such specimens and the potential obligation to inform if the

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new test provides information that may potentially have a fundamental impact on that individual if discovered. In general, if personal identifiers are linked to the specimen, the investigator may have the duty to inform regardless of whether the specimen was initially obtained and studied for the same or different purpose. Patient contact should be done under IRB–approved protocol only.

Table 3 and Table 4 illustrate the method used to calculate the (clinical) diagnostic sensitivity and specificity and to determine the confidence limits for the estimates, respectively.

**Table 3. Diagnostic Sensitivity and Specificity**

| | | Disease Status | | |
|---|---|---|---|---|
| | | Positive (D+) | Negative (D-) | |
| Assay Results | Test Positive | a | b | a+b |
| | Test Negative | c | d | c+d |
| | | a+c | b+d | |

KEY: assay/disease

| | | |
|---|---|---|
| a = | +/+ | true positive |
| b = | +/- | false positive |
| c = | -/+ | false negative |
| d = | -/- | true negative |

Diagnostic sensitivity = a/(a + c), and
Diagnostic specificity = b/(b + d)

Confidence intervals may be calculated using the exact binomial method. The following table illustrates the confidence interval around the estimates of sensitivity/specificity for various sample sizes and test outcomes.

**Table 4. 95% Confidence Intervals on Estimate**

| Sample Size (a+b) | Sensitivity/Specificity Estimates (p) | | | |
|---|---|---|---|---|
| N | 0.60 | 0.85 | 0.90 | 0.98 |
| 20 | [0.36-0.81] | [0.62-0.97] | [0.68-0.98] | [0.85-0.999] |
| 50 | [0.46-0.74] | [0.75-0.95] | [0.82-0.98] | [0.89-0.999] |
| 100 | [0.50-0.70] | [0.78-0.92] | [0.84-0.96] | [0.95-0.998] |
| 200 | [0.53-0.67] | [0.80-0.90] | [0.86-0.94] | [0.95-0.995] |

11.5.2.5 Predictive Values (PV)

Positive predictive values (PV+) and negative predictive values (PV-) are key to evaluating the effectiveness of a test in the target population(s), especially for tests intended for broad-based population screening. The predictive values are a function of the prevalence of disorder in the relevant population and the diagnostic sensitivity and specificity of the test. In order for the predictive values to be adequately estimated, the sensitivity and specificity should be estimated with sufficient accuracy. Predictive values may be difficult to calculate accurately for rare disorders of unknown frequency.

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PV+ = P(D+|T+) = 1 – false-positive rate, and
PV- = P(D-|T-) = 1 – false-negative rate.

Where T = test result,
D = disease/state of interest, and
P(D+) = prevalence of disease or state of interest (i.e., probability that the disease or state of interest is present in the target population).

### 11.5.3 Limitations

One should identify any limitations and contraindications for use of the test, including factors that impact adversely on accuracy of test interpretation (e.g., allelic mutations that cannot be detected by the test, less than optimal analytic performance, etc.) and any technical limitations of the assay.

### 11.5.4 Test Results

Correct interpretation of test results requires knowledge of the target locus/allele/mutation characteristics of the disorder in question, the analytic and diagnostic performance properties of the test, intake information, etc. In some cases, "validation" of test interpretation may require evaluation by a panel of experts.

Analysis and interpretation of data should take into consideration how equivocal results will be interpreted. An appropriate response should be established for an equivocal result; e.g., repeat specimen, repeat test, test reporting, etc.

### 11.5.5 Verification/Internal Validation of Established Procedure

Laboratories using commercially available devices according to manufacturers' instructions (devices already validated by the manufacturer) should perform appropriate studies to verify that performance properties established by the manufacturer are obtained in the laboratory setting. Laboratories that modify commercially available devices should fully validate the modified device's performance.

Laboratories using in-house-developed procedures may establish the performance parameters by verifying another laboratory's performance, provided the procedure has undergone full validation by the other laboratory. This may be accomplished retrospectively (blinded testing) or by splitting samples prospectively.

## 11.6 Postvalidation/Routine Use Quality Assurance

**NOTE:** Laboratories using FDA-approved commercial probe or nucleic acid amplification test kits should follow the QA procedures outlined by the manufacturer. Laboratories may choose to add additional controls, depending on the number and types of samples processed by the laboratory.

If modifications are made to a protocol of a commercial test, or if a test is used on specimen types other than those specified by the manufacturer, validation studies, as described in the previous section, must be performed before results are reported on clinical samples. Additional positive and negative controls to ensure test performance will likely be necessary.

### 11.6.1 Postvalidation Procedures

The QA program of a laboratory that performs hybridization assays involving nucleic acid probes or nucleic acid amplification begins with the development of a comprehensive quality control (QC) program. Controls to ensure proper performance of enzymes, reagents, and equipment are critical to producing

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accurate results. However, QC does not end when the assay is complete. Controls also must be in place to ensure that the laboratory reports the correct results to the appropriate laboratory or physician. Because of the sensitivity of these methods, the types, number, and placement of controls within an assay are crucial to the detection of contamination (false-positive results) and the presence of inhibitors in samples (false-negative results). The use of adequate numbers and types of controls is particularly important for laboratories that are just beginning to implement probe or amplification assays.

A key element in QC that is often overlooked is laboratory design. Preventing crossover contamination is easier if there is adequate space available to separate personnel involved in reagent preparation, sample processing, and assay performance. While many laboratories may not have separate rooms available to devote to these functions, much can be done to reduce contamination by judicious location of personnel, use of hoods, and strict attention to specimen flow in the laboratory. It is wise to remember that laboratories that have been processing specimens for many years are likely to be highly contaminated before the first hybridization or amplification reaction is performed. Therefore, it is highly recommended that any space used for these methods be thoroughly cleaned and disinfected before use.

### 11.6.2 Procedure Manuals

Manuals that detail each hybridization and nucleic acid amplification procedure and all QC procedures must be in written form and available within the laboratory. They should include sections on laboratory safety. Each manual should be reviewed and signed by all personnel working in the laboratory, and each method should be reviewed annually by the laboratory supervisor. Quality control procedures should define what constitutes an “in-control” and an “out-of-control” result. A manual detailing protocols for specimen collection and transport to the laboratory should be distributed to all hospital wards, laboratories, and clinics that are likely to submit specimens for analysis. The manual should include a list of the assays available and the expected turnaround times for those assays.

### 11.6.3 Reagent Quality Control Program

Quality control of reagents is a critical aspect of all laboratory programs, but it is especially important in hybridization and nucleic acid amplification, because contamination can so easily invalidate assay results. All nucleic acid reagents and enzymes should be tested for concentration, purity, and functionality. Aliquot reagents should be stored as appropriate. This should be done before new reagents are used in assays with clinical samples. Results obtained using those new reagents should be compared to those generated using current reagents in crossover studies and proven to be comparable. Concentration of primers and probes can be checked by absorbance at 260 nm, while purity can be confirmed using polyacrylamide-gel electrophoresis. Functionality can be checked in assays using target DNA concentrations near the lower limit of detection (see Table 5).

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**Table 5. Quality Assurance of Test Component Reagents**

| Reagent | Test | Frequency | Rejection Criteria |
|---|---|---|---|
| Oligonucleotide primers and probes | Purity: absorbance 260/280 | Each new lot | If absorbance is <1.6, repurify. |
| | Purity: acrylamide gel | Each new lot | Reject if more than one band per oligo or if wrong size. |
| | Contamination check: 5-10 negatives (primers) | Each new lot | Reject if any positives are detected. |
| | Functional check (parallel with old lot) | Each new lot | Reject or adjust concentration if performance is significantly different from the proven lot. |
| Buffers, deoxyribonucleoside triphosphates | Functional check (parallel with proven lot) | Each new lot | Reject or adjust if performance is significantly different from the proven lot. |
| Restriction endonucleases | Digest plasmid with established pattern | Each new lot | Reject if digestion pattern is different than established. |
| Enzymes (i.e., amplification, ligation) | Functional check (parallel with proven lot) | Each new lot | Reject or adjust if performance is significantly different than the proven lot. |

### 11.6.4 Control Parameters for Clinical Assays

**NOTE:** Controls that include sample or specimen matrix effects should be considered during assay development, although these are sometimes difficult to achieve for routine use (see Table 6).

#### 11.6.4.1 Positive Controls

Positive controls should be present in each assay run. The concentration of the control analyte should be in the low range of the assay and should challenge the sensitivity of the assay. Positive controls should be at low levels so as not to serve as a source of crossover contamination.

#### 11.6.4.2 Negative Controls

Negative controls consist of all assay reagents and nontarget nucleic acid. These are used to detect crossover contamination and should be used frequently within runs to detect contamination. For amplification-based test methods that have been developed in-house (where contamination issues have not been fully eliminated), at least every fifth tube should be a negative control.

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**Table 6. Test Controls**

| Assay Component | Control Used | Frequency | Rejection Criteria |
|---|---|---|---|
| Specimen preparation | Sample presentation, positive and negative | Each sample preparation run | Reject if negative control is positive (contamination) or if positive control is negative (inefficient sample preparation). |
| Specimen preparation/ amplification | Template control: qualitative indicator | Each specimen | Reject specimen result if negative (nonamplifiable specimen due to inhibitor, low template copy number, instrument incapabilities). Repeat, or repeat at lower dilution; try more vigorous extraction. |
| Amplification | Low-positive target and negative target | Each amplification run | Repeat if negative control is positive (contamination or nonspecific amplification) or if positive control is negative (no amplification). |
| Detection | Target or diluted amplified target and negative | Each detection run | Reject if negative control is positive (detection carry-over due to technique or apparatus; cross-detection due to decrease in stringency). Reject if positive control is negative (detection not working; reagents missing, inactive or too stringent). |
| | Reagent blanks (when method warrants) | Each detection run | Reject if blanks are too high as per procedure. |

**NOTE:** The control strategies listed above are recommended for laboratories performing tests that were developed on-site or are modifications of FDA-cleared or FDA-approved commercial kits. The selection, placement, and frequency of these controls will depend on the assay system, the degree of development and standardization of that system, and a particular laboratory's experience with the system. Laboratories performing tests from commercial kits should follow the manufacturers' instructions or the instructions of other accrediting agencies.

11.6.4.3 Amplification and Inhibitor Controls

Two key aspects of quality control are: ensuring that there is sufficient nucleic acid present in an amplification assay to produce a positive result if the target sequence is present; and ensuring that there are no inhibitors present in a sample that would produce a false-negative result. To control for each of these problems, samples can be split and analyzed in duplicate. To one aliquot of the sample, an alternate primer pair and target DNA is added to detect inhibitors that may be present (see Section 9.4.1). In the second assay, a second primer pair that targets endogenous nucleic acid sequences, such as the genes for HLA-DQA1 or β-globin, is used to indicate the presence of amplifiable target nucleic acid in the sample. For assays involving bacteria or other micro-organisms, a second primer pair from a target site such as a gene specifying ribosomal RNA may be used. Note, however, that some problems with exogenous DNA

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contaminating *Taq* polymerase have occurred. Thus, primers to highly conserved sequences must be chosen with caution.

### 11.6.5 Quality Control of Equipment

All equipment should be sterilized and preventive maintenance should be performed routinely. Quality control charts that indicate acceptable temperature ranges should be posted on all refrigerators, freezers, water baths, incubators, and heating blocks. Temperatures should be checked and recorded on these charts daily. When an instrument is found to be out of range, the temperature should be circled in red ink, and the corrective action taken should be recorded. Procedures for instrument calibration should be in the laboratory's QA manual, and they should be reviewed annually. Table 7 summarizes the QA of equipment.

#### 11.6.5.1 Pipette Calibration

Pipettors should be calibrated at least twice a year to ensure accurate delivery of reagents. Calibration should be performed with the type of tips most commonly used in the laboratory. For example, if plugged pipette tips are used routinely, then these also should be used for calibration studies.

#### 11.6.5.2 Thermal Cyclers

There are many different types and models of thermal cyclers available. Each well of a thermal cycler should be tested at least twice a year to ensure uniform heating throughout the block. Ramping times are a critical aspect of many assays and should be checked by printing the cycle parameters.

#### 11.6.5.3 Centrifuges

Centrifuges can serve as a major source of aerosols and contamination if samples are not properly loaded and spun in sealed containers. The speed of the centrifuge should be checked at least twice a year. The instrument should be cleaned on a regular basis with freshly prepared 5 to 10% bleach and specimen holders checked for cracks and wear.

#### 11.6.5.4 Biological Safety Cabinets

Contamination is a key source of erroneous results in the nucleic acid amplification laboratory; therefore, it is suggested that separate safety cabinets or hoods be designated for reagent preparation and for sample processing. Hoods should be decontaminated at the start and end of each work day and immediately after a spill or accident. The airflow in all safety cabinets and hoods should be monitored continuously and calibrated at least annually. The placement of ultraviolet (UV) lights in hoods may decrease the contamination of samples by inactivating DNA, but it also poses an additional safety hazard to workers. Therefore, UV lights should be used with caution. Also, the energy efficiency of UV lights should be checked at least annually.

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**Table 7. Instruments and Equipment**

| Instrument/ Equipment | Procedure | Frequency | Rejection Criteria |
|---|---|---|---|
| Thermal Cyclers* | Instrument diagnostic tests | Each time instrument is moved and as per manufacturers' instructions. | Reject upon failure of any test; call manufacturer. |
| | Temperature monitoring with thermocouple | Monthly for high volume | Reject if target temperatures are not within instrument specifications or if temperature variation across block is out of specification. Call manufacturer (can avoid specific wells that are out of range). |
| | Functional amplification check (low copy amplification in all wells) | Quarterly for high volume | Temperature check wells not amplifying as predicted, then follow temperature-check decisions. |
| | Program printout | Each run for diagnostic tests | Reject if printout reveals that the wrong program was used or the amplification program was altered. |
| Pipettors | Calibration | Two times per year | Do not use if out of specification. Refer to laboratory procedures. |
| Water baths, heating blocks | Temperature check | Each use | Do not use if out of specification. Refer to laboratory procedures. |
| Spectrophotometers, fluorometers, luminometers | Preventative maintenance Calibration | Two times per year | Do not use if out of specification on calibration. Recalibrate or call manufacturer. Refer to laboratory procedures. |
| Microscopes | Preventative maintenance | Annually | |

*Frequency of these tests depends on the historical reliability of the instrument used and the standardization and robustness of the assay being run.

11.6.5.5 Spectrophotometers and Luminometers

Spectrophotometers and luminometers are frequently used in conjunction with nonradioactive reporter systems. The instruments should undergo preventative maintenance at least yearly and be calibrated at least every six months to ensure accuracy.

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### 11.6.6 Recording and Reporting of Results

Although probe and amplification assays themselves may be tightly controlled, erroneous results may still be reported if a system is not in place to screen for clerical and reporting errors. Supervisors should review all results before the reports leave the laboratory.

#### 11.6.6.1 Notification of Inadequate Samples

If the laboratory receives samples that are unacceptable for analysis, the requesting laboratory or physician must be notified promptly. Samples should be rejected (after notifying the requesting laboratory or physician) if they have been transported improperly, delayed in transport for significant amounts of time, are not preserved, or are of insufficient volume.

#### 11.6.6.2 Preliminary Reports

Preliminary reports should be issued, if appropriate, to indicate what work was accomplished and what results are pending.

#### 11.6.6.3 Final Reports

Final reports should be issued only after supervisory review. Final reports that conflict with preliminary reports should be investigated to determine the source of error.

#### 11.6.6.4 Turnaround Time

Procedures for monitoring the turnaround time of results should be in place in the laboratory to detect potential problems that delay the reporting of results.

### 11.6.7 Recording and Reporting of Quality Control Data

#### 11.6.7.1 Logbooks

All QC data should be recorded in logbooks and reviewed at least monthly by the laboratory supervisor. The supervisor should initial the logbooks after reviewing the data. Out-of-control results should be circled in red ink and corrective actions recorded in the log. Consistent problems should be noted. Lot numbers and the expiration dates of all reagents and kits should be recorded in logbooks.

#### 11.6.7.2 Reagents

The receipt date and expiration date of reagents should be noted on each reagent bottle or kit that is received in the laboratory. Reagents from different manufactured kit lot numbers should not be interchanged.

### 11.6.8 Selection of Referral Laboratories

Most laboratories send some types of specimens to other laboratories for testing. For guidance on the selection of a referral laboratory, consult the current version of NCCLS document GP9—*Selecting and Evaluating a Referral Laboratory*.

## 11.7 Proficiency Testing

Proficiency tests are used to ensure the reproducibility of clinical tests and to confirm the skill of a clinical or referral laboratory in performing such tests. Proficiency testing may be sponsored by local,

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regional, or national organizations. Proficiency testing is now required of every laboratory that assays clinical samples, and it is used to determine which laboratories qualify to perform specific clinical tests. These tests permit the evaluation of the capacity of the laboratory to derive results that are in agreement with those of other laboratories using the same methodology. The results of proficiency testing also permit assessment of how results from one method agree with results derived using other methods, assuming that the proficiency testing materials are compatible with procedures that are different from those that were originally developed.

Each clinical laboratory is required to enroll in an approved program or programs for each of the specialties, subspecialties, analytes, or tests for which it seeks certification. Unfortunately, there are few approved proficiency testing programs that include testing using molecular biology techniques; however, additional programs are currently under development.

Proficiency testing (PT) for many analytes is available through professional organizations.[e] If no pre-established or suitable PT program is available for a particular assay, laboratories should design their own sample exchange program.

When establishing such a program, several issues should be considered. The PT provider should be a practicing expert in the field. Exchange of at least four coded samples per year is advisable; and each shipment should be accompanied by a form to document the date of testing, methods used, and results obtained. A performance report is generated by the PT provider. It goes without saying that the more laboratories that participate and the more samples that are exchanged, the stronger the statistical power of the results and the easier it is to resolve discrepancies. Laboratories using comparative methods are most likely to have comparable results. For purposes of clinical correlation, the PT provider should collect corroborating clinicopathologic evidence for or against the disease or condition relating to the analyte in question. The PT program should establish procedures to help participants identify deficiencies causing PT failure and to help determine whether such deficiencies have been corrected. In the rare circumstance whereby no laboratory is available with whom to establish a sample exchange program, blind retesting of samples with known results can be used as an alternate, albeit less desirable option.[3]

### 11.7.1 Frequency and Components of Proficiency Testing

Annual proficiency testing programs should follow regulatory requirements. We recommend that a program should provide a minimum of four test samples per year, and there should be at least two testing events at approximately equal intervals per year. Provided samples must cover the full range of reactivity, from highly reactive to nonreactive. Samples should be examined or tested with the laboratory's regular patient work load, by personnel who routinely perform the testing and in the same manner as the laboratory routinely tests patient specimens.

### 11.7.2 Specimens for Proficiency Testing

Proficiency testing specimens are normally of the same type or similar in composition to those normally tested in the clinical laboratory (e.g., whole blood, biopsy materials), and they should be fully characterized for the presence of the molecular target for which they are being tested, using all methods available, before being distributed. These specimens are ordinarily obtained from outside sources; however, this is not always possible. While these may resemble patient specimens, the nucleic acids present may be in a form not usually found in clinical specimens and may not work in a particular assay. The skills of the technologist performing the assay, the technique employed, and the processing and handling of the specimen in the user laboratory may produce varying results.

[e] Such as the College of American Pathologists (CAP).

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### 11.7.3 Sources of Nucleic Acids Used in Proficiency Testing Specimens

The source of the nucleic acids used in proficiency testing for molecular diagnostics is a critical factor in testing the results obtained. The following factors should be considered:

#### 11.7.3.1 Cultured Cells Containing Known Copy Numbers of Genomic Equivalents

Sufficient quantities of well-characterized, donor-supplied, reactive specimens may sometimes be unavailable. In such situations, the use of cultured cells from cell lines containing genomic equivalents of the agent at known copy numbers may sometimes be added back to nonreactive specimens to produce specimens with varying degrees of reactivity. This technique is particularly useful in producing proficiency specimens where the nucleic acids tested are purified from whole cells, such as lymphocytes or epithelial cells, but it should not be used if the specimen is a cell-free body fluid such as serum.

#### 11.7.3.2 Cloned Nucleic Acid Sequences and Nucleic Acids Isolated from Purified Organisms

Cloned nucleic acid sequences should contain only sequences which are being tested. Inclusion of vector or nontarget nucleic acid sequences in proficiency specimens may result in nonspecific reactivity in hybridization and amplification reactions. If vector or nontarget nucleic acid sequences are included in proficiency specimens, noninterference should be validated. If vector or nontarget nucleic acid sequences are included in proficiency specimens, validation must prove that they do not affect test specificity. When producing proficiency specimens by the addition of cloned or purified nucleic acid sequences to nonreactive specimens, consideration should also be given to the stability of the nucleic acids in the specimen due to the presence of nucleases and other nucleic-acid-modifying enzymes. Agents used to stabilize specimens should be selected so as not to interfere with assay conditions.

### 11.7.4 When There Are No Sources of Proficiency Testing Materials

Whenever proficiency testing programs or proficiency specimens are unavailable for specific tests, laboratories should implement procedures to test for internal consistency in their results. This may be accomplished by testing duplicate or split specimens from individual persons, with one-half being tested under the patient's name and the other half identified in a manner that obscures the source of the specimen. Blinding as to the source of the specimens may be accomplished in several ways, but it should always be done so that the technologist performing the assay cannot relate a blinded specimen to a specimen from a particular patient. Both the patient-identified specimen and blinded specimen should be randomly tested by technologists to reduce bias. The results obtained from the two specimens should always be in agreement.

The following methods should be used to validate the results of this type of testing:

#### 11.7.4.1 Crossover Specimens from Previous Panels

Previously characterized specimens or specimens used in previous internal consistency tests should be included in proficiency test runs.

#### 11.7.4.2 Performing Other Testing Methods

Characterization of a particular testing technique may have its sensitivity and specificity validated by testing the same specimens using other procedures such as immunological testing or other molecular biology techniques.

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11.7.4.3 Sending Specimens to Other Laboratories

In the absence of approved proficiency testing programs, testing for internal consistency with molecular biology techniques may be accomplished by sending tested and characterized specimens to outside laboratories to be tested using the same techniques. This practice should only be used to validate testing procedures, and it should be discontinued once approved proficiency programs become available.

### 11.7.5 Documentation and Recordkeeping

Laboratories should carefully document all steps in the testing and reporting of results for all proficiency samples, including the handling, processing, and examination of the specimens. Laboratories should retain copies of these records, including all report forms required by the proficiency testing programs and by federal, state, and local stature for a minimum of two years from the date of the proficiency-testing event. However, precedent suggests that there is no safe time to dispose of records, because testing "results [may] be sought as evidence in civil or criminal litigation."

## 12 Gene Rearrangement Assays

Gene rearrangement assays are designed to facilitate the identification of an expanded monoclonal cell population within a population of lymphoid cells. The detection of such a population is a useful adjunct in attempting to establish a diagnosis of malignancy, and is useful in helping to establish the lineage of lymphoid neoplasms. Nevertheless, identification of a monoclonal cell population is not a perfectly reliable indicator of either malignancy or lineage.

## 12.1 Immunoglobulin Heavy Chain Rearrangement

To date, the only amplification-based assays for immunoglobulin heavy chain rearrangement are PCR-based. Following amplification, products are electrophoretically separated and visualized.

### 12.1.1 Preparation and Amplification

Because the ability of the assays to detect a monoclonal population depends in part upon the fraction of the cell population that demonstrates the rearrangement, the sensitivity of gene rearrangement assays may sometimes be improved by microdissection and amplification of a morphologically suspicious cell population.[4] In addition, the detection of rearranged bands may depend on the total quantity of target DNA present in the specimen.[5] Thus, particularly when formalin-fixed, paraffin-embedded tissues are used for analysis, it is prudent to perform several assays, including a range of DNA target amounts (dilutions).[5,6]

In general, immunoglobulin heavy chain assays are most reliable when they are designed to give rise to a product with a length of between 105 and 120 base pairs. Because nonspecific priming may cause both an increase in nonspecific background bands and a decrease in the intensity of a true monoclonal rearrangement band, the use of a method (such as hot start) to reduce the effects of nonspecific priming is highly recommended.

Careful attention to primer design is very important. Although no PCR-based method is capable of detecting all rearrangements that may be observed by Southern blot, the sensitivity of approaches reported in the literature varies widely when applied to well-characterized samples. In general, the use of degenerate consensus V-region primers is required to achieve optimal results. Some commonly used primer combinations are shown in Table 8. The primer set shown is able to detect approximately 72% of all possible heavy chain rearrangements.[7] Typically, the PCR-based assays are able to detect a monoclonal population of approximately 20% to 25% when diluted into polyclonal lymphoid cells.[6,7] Increased sensitivity, achieved at the price of increased technical complexity, may be achieved by

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incorporating larger numbers of V-region primers into the reaction mix. Assays based upon such complex reaction mixes may be able to detect more than 90% of possible heavy chain rearrangements.[8,9]

**Table 8. A Typical Primer Pair for Immunoglobulin Heavy Chain Rearrangement Assays**

| Region | Designator | Sequence |
|---|---|---|
| V | FR3A | 5' ACA CGG C(C/T)(G/C) TGT ATT ACT GT 3' |
| J | CFW1 | 5' ACC TGA GGA GAC GGT GAC CAG GGT 3' |

### 12.1.2 Electrophoresis

Although early publications suggested the use of agarose gels for electrophoresis,[10] the relatively poor resolution of agarose-gel electrophoresis has caused most laboratories to discontinue their use. At this time, polyacrylamide-gel electrophoresis is used by most laboratories, and is strongly recommended.[6,11] The percentage polyacrylamide varies widely from laboratory to laboratory, but there are no data that suggest substantial differences in performance among laboratories using different polyacrylamide gels. In some laboratories, denaturing gels and/or single-strand conformational polymorphism analysis is employed as part of the analytical scheme.[11,12] These techniques may increase detection sensitivity, but experience with them is limited to relatively few laboratories.

An alternative to the use of traditional polyacrylamide gel electrophoresis plates is capillary electrophoresis. Commercially available capillary electrophoresis systems make use of fluorophor-labeled primers which are electronically detected as the electrophoresis takes place, eliminating the need for a separate visualization step.

### 12.1.3 Visualization

Visualization may be accomplished by incorporating a radioactive label into the PCR mix, by use of radiolabeled, biotinylated, or digoxigenin-labeled primers followed by chemiluminescent visualization, or by ethidium bromide staining. While the subcommittee members have the impression that ethidium bromide visualization may be somewhat less sensitive than other methods, hard data are lacking.

### 12.1.4 Interpretation

Regardless of visualization method, methods employing traditional flat gel electrophoresis give rise to bands and band patterns that may, at times, be difficult to interpret. Laboratories should establish a range of amplicon sizes within which it is considered permissible to interpret the presence of a strong band as “monoclonal." Even strong bands with a size outside this range should not be given an unequivocal interpretation as “monoclonal." Where possible, laboratories should develop quantitative criteria for both the sharpness and band strength required for the laboratory to interpret a band as “monoclonal." Moreover, duplicate (or replicate) samples should be performed, and only those with identical bands should be considered indicative of monoclonality.

Capillary electrophoresis devices generally show a series of bands. The reporting format makes the development of quantitative criteria, and the interpretation of the electrophoregram, more straightforward than for flat gel electrophoresis.

### 12.1.5 Reporting

Laboratories should make available to the pathologist or clinician information regarding the sensitivity and specificity of their assays. This information may be included in information for ordering the test, or as

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a parenthetical note on the test report. Such a statement should state the theoretical fraction of possible immunoglobulin gene rearrangements that can be identified, a practical indication of the fraction of B-cell neoplasms that are identified (preferably based upon side-by-side comparison with Southern-blot results), and a statement of the percentage of cells that must belong to a monoclonal population to visualize it in the assay. If results are not available from within the laboratory, literature references for this data are acceptable.

Certain immunoglobulin gene rearrangement assays fail frequently to identify a monoclonal population in specific lymphoid neoplasms (the most common failure is the identification of rearrangement in follicular lymphoma).[13,14] When this is known to be the case for a particular assay, this must be conveyed to the clinician via either the test ordering information or a parenthetic note on the report, if applicable.

### 12.1.6 Limitations

Limitations may include the following:

- No currently available amplification-based assay is capable of identifying all rearrangements that may be observed by Southern blot. It is therefore advisable that Southern blot be available to the laboratory as a supplemental method.

- As noted above, certain gene rearrangement assays fail frequently to identify specific neoplasms, most commonly follicular lymphoma. Therefore, many laboratories recommend bcl-2/JH translocation studies to complement heavy-chain gene studies in diagnostic testing.

- 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 proliferations can occur, particularly in immunocompromised patients or patients with certain viral infections.

- Occasionally, T-cell neoplasms demonstrate rearranged immunoglobulin genes. In addition, myeloid neoplasms can also demonstrate immunoglobulin gene rearrangement.[15] This phenomenon is termed “lineage infidelity." Persons making use of gene rearrangement assay data must be aware of this phenomenon.

### 12.1.7 Controls

The proper choice of positive-assay controls for gene rearrangement assays is often difficult. The ideal positive assay control is derived from a tissue typical of that seen within the laboratory, and has only a barely detectable number of rearranged cells. Such controls are almost never available, and when available, are rapidly and irreplaceably depleted. An alternative, commonly employed control consists of rearranged cells from a cell line, such as the Raji Burkitt lymphoma cell line, diluted into peripheral blood lymphocytes that the cell line contributes approximately 20 to 25% of total DNA. Such a mixture can be preserved frozen, fixed in ethanol, or prepared as a cell block, so that appropriately prepared controls are available for several different types of specimens.

A housekeeping gene is typically used as a control to demonstrate amplifiability of sample DNA. Any different housekeeping genes are in common use, including interferon-γ, neu, β-globin, and others. Although the control gene can reflect any genomic target, the amplification used as a control should result in a product similar in size to the product anticipated for the rearranged immunoglobulin- or T-cell-receptor gene.

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## 12.2 T-cell Receptor Rearrangement

### 12.2.1 General Considerations

In general, PCR-based approaches to detection of T-cell receptor gene rearrangements in leukemias and lymphomas rely upon the identification of changes in either the β[16,17] or γ[18,19] chain genes. In some ways, assays for γ chain rearrangement are technically simpler, and are currently employed by a majority of laboratories. Nevertheless, there is a limited combinatorial diversity for this receptor, so separation on the basis of sequence, as well as by size, is generally recommended; this is accomplished most readily by use of single-strand conformational polymorphism or denaturing gradient gel electrophoresis techniques. Although PCR-based techniques are not capable of detecting as wide a range of rearrangements as Southern blot methods,[20] they may identify the presence of monoclonal populations in specimens that are insufficiently cellular for Southern-blot analysis, such as many skin biopsies.[21]

### 12.2.2 Preparation and Amplification

Because the ability of the assays to detect a monoclonal population depends in part upon the fraction of the cell population that demonstrates the rearrangement, the sensitivity of gene rearrangement assays may sometimes be improved by microdissection and amplification of a morphologically suspicious cell population.[4] In addition, the detection of rearranged bands may depend on the total quantity of target DNA present in the specimen.[5] Thus, particularly when formalin-fixed, paraffin-embedded tissues are used for analysis, it is prudent to perform several assays, including a range of DNA target amounts (dilutions).[5,6] While most evaluations of experimental conditions have focused on immunoglobulin heavy-chain rearrangement, the same considerations are expected to apply for the T-cell receptor genes.

In general, gene rearrangement assays are most reliable when they are designed to give rise to a product with a length of between 105 and 120 base pairs. Because nonspecific priming may cause both an increase in nonspecific background bands and a decrease in the intensity of a true monoclonal rearrangement band, the use of a method (such as HotStart®) to reduce the effects of nonspecific priming is highly recommended.

### 12.2.3 Electrophoresis

Although early publications suggested the use of agarose gels for electrophoresis,[10] the relatively poor resolution of agarose-gel electrophoresis has caused most laboratories to discontinue their use. At this time, polyacrylamide-gel electrophoresis is used by most laboratories, and is strongly recommended.[6,11] The percentage polyacrylamide varies widely from laboratory to laboratory, but there are no data that suggest substantial differences in performance among laboratories using different polyacrylamide gels. In many laboratories, denaturing gels and/or single-strand conformational polymorphism analysis is employed as part of the analytical scheme, and there is abundant evidence that they increase the detection sensitivity for T-cell clones.

An alternative to the use of traditional polyacrylamide gel electrophoresis plates is capillary electrophoresis. Commercially available capillary electrophoresis systems make use of fluorophor-labeled primers which are electronically detected as the electrophoresis takes place, eliminating the need for a separate visualization step.

### 12.2.4 Visualization

Visualization may be accomplished by incorporating a radioactive label into the PCR mix, by use of radiolabeled, biotinylated, or digoxigenin-labeled primers followed by chemiluminescent visualization, or by ethidium bromide staining. While the subcommittee members have the impression that ethidium bromide visualization may be somewhat less sensitive than other methods, hard data are lacking.

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### 12.2.5 Interpretation

Regardless of visualization method, methods employing traditional flat-gel electrophoresis give rise to bands and band patterns that may, at times, be difficult to interpret. Laboratories should establish a range of amplicon sizes within which it is considered permissible to interpret the presence of a strong band as "monoclonal." Even strong bands with a size outside this range should not be given an unequivocal interpretation as "monoclonal." A positive result may be seen in B-cell lymphoblastic lymphoma/leukemia, myeloid leukemia, lymphocytic thymoma, and in cases in which the number of T cells present in the sample is small. A false-negative result may occur if the gene rearrangements lead to interference with the primer binding. Where possible, laboratories should develop quantitative criteria for both the sharpness and band strength required for the laboratory to interpret a band as "monoclonal." Moreover, duplicate (or replicate) samples should be performed, and only those with identical bands should be considered indicative of monoclonality.

Capillary-electrophoresis devices generally show a series of bands. The reporting format makes the development of quantitative criteria, and the interpretation of the electrophoregram, more straightforward than for flat-gel electrophoresis.

### 12.2.6 Reporting

Laboratories should make available to the pathologist or clinician information regarding the sensitivity and specificity of their assays. This information may be included in information for ordering the test, or as a parenthetic note on the test report. Such a statement should state the theoretical fraction of possible T-cell receptor gene rearrangements that can be identified, a practical indication of the fraction of T-cell neoplasms that are identified (preferably based upon side-by-side comparison with Southern-blot results), and a statement of the percentage of cells that must belong to a monoclonal population to visualize it in the assay. If results are not available from within the laboratory, literature references for this data are acceptable.

### 12.2.7 Limitations

Limitations may include the following:

- No currently available amplification-based assay is capable of identifying all rearrangements that may be observed by Southern blot. It is therefore advisable that Southern blot be available to the laboratory as a supplemental method.

- 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 proliferations can occur, particularly in immunocompromised patients or patients with certain viral infections. Monoclonal T-cell receptor gamma chain rearrangements have been observed in drug-associated cutaneous eruptions associated with dermal-lymphoid proliferation processes.[22] Clonal T-cell receptor gene rearrangements have been described in several disorders that are not necessarily malignant such as lymphomatoid papulosis, large granular lymphocytosis, PLEVA, and even normal thymus.

- Occasionally, B-cell neoplasms demonstrate rearranged T-cell receptor genes. In addition, myeloid neoplasms can also demonstrate T-cell receptor gene rearrangement. This phenomenon is termed "lineage infidelity." Persons making use of gene rearrangement assay data must be aware of this phenomenon.

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### 12.2.8 Controls

The proper choice of positive assay controls for T-cell receptor rearrangement assays is often difficult. The ideal positive assay control is derived from a tissue typical of that seen within the laboratory, and has only a barely detectable number of rearranged cells. Such controls are almost never available, and when available, are rapidly and irreplaceably depleted. An alternative, commonly employed control consists of rearranged cells from a cell line diluted into peripheral blood lymphocytes to which the cell line contributes approximately 20 to 25% of total DNA. Such a mixture can be preserved frozen, fixed in ethanol, or prepared as a cell block, so that appropriately prepared controls are available for several different types of specimens.

A housekeeping gene is typically used as a control to demonstrate amplifiability of sample DNA. Any different housekeeping genes are in common use, including interferon-γ, neu, β-globin, and others. There is no obvious advantage to any particular choice.

## 13 DNA-based Translocation Assays

### 13.1 General Considerations

PCR-based translocation assays are based upon analysis of the ribonucleic acid transcript or are carried out on cellular DNA. The technical methodology is generally straightforward, and adequately covered by other portions of these guidelines. Nevertheless, each assay has some unique characteristics and limitations. Some of the more important assay-specific features are considered below for two of the more commonly used PCR-based translocation assays.

### 13.2 bcl-1 Rearrangement

The t(11;14) bcl-1/IgH rearrangement is identified by PCR in about half of patients with mantle cell lymphoma.[21-25] The relatively low sensitivity limits the diagnostic utility of this assay.[24] Although the assay may be used to assess the presence of minimal residual disease in whose tumors bear the translocation,[26,27] the clinical conditions under which this is useful have not been delineated.

### 13.3 bcl-2 Rearrangement

The t(14;18) bcl-2/IgH rearrangement is most frequently associated with follicular lymphoma. Nevertheless, this rearrangement has also been reported in other non-Hodgkin's lymphomas,[28] Hodgkin's disease,[27-31] and reactive lymphoid hyperplasia.[32,33] The types of disease in which the translocation is identified by PCR methods depends upon the details of the PCR assay. "Standard" PCR assays do not identify cells bearing t(14;18) in reactive lymphoid hyperplasia, for example, while those that have been "extreme-sensitivity achieved" by such techniques as nesting, frequently do.[33,34] Clearly, therefore, great care is required in the interpretation of assay results, and in assessment of clinical utility.

Assays for t(14;18) are one method for the identification of "minimal residual disease" in lymphoma. Lymphoma patients in whom cells bearing the t(14;18) can be cleared have a substantially better survival than those in whom residual t(14;18) bearing cells can be identified following treatment.[33-37] Nevertheless, patients have been reported to harbor cells bearing t(14;18) in the peripheral circulation for many years without developing clinical evidence of disease.[38,39]

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# 14 Mutation Assays

## 14.1 General Considerations

Assays intended to determine the presence and nature of point mutations are for detection and management of acquired hematologic disease, but are restricted to hereditary diseases. Because the identification of a hereditary genetic abnormality carries implications far beyond those associated with the treatment of a specific patient's disease, laboratories performing genetic testing have special obligations which must be fulfilled. Many of these special obligations are considered in NCCLS document MM1—*Molecular Diagnostic Methods for Genetic Diseases*. In this section, we will discuss a few of the issues specific to testing for three of the more common inherited hematologic diseases. Other complex diseases, including hemophilia B and the thalassemias will not be discussed.[f]

## 14.2 Factor V Leiden

Some mutations in Factor V result in hemorrhagic diathesis, while others predispose to deep venous thrombosis. The most common mutation predisposing to thrombosis, a 1691 G → A mutation commonly referred to as Factor V Leiden, has allele frequency of 6 to 8% in the Caucasian population.[40] Prevalence is lower in African Americans, Hispanic Americans, Asian Americans, and Native Americans, but is comparable in these populations to the allele frequency of variant prothrombin in Caucasians.[41] Mutation results in resistance of the activated form of Factor V to degradation by activated protein C (APC), and is associated with a three- to sixfold increase in deep venous thrombosis risk when present as a single allele;[40,42] patients who have two copies of Factor V Leiden have a risk approximately fifty- to one hundredfold higher than those with wild-type Factor V genes.

The conditions under which testing for the Factor V Leiden mutation are appropriate have not been completely developed; testing does not appear to be warranted for the relatives of patients with thromboembolic events demonstrated at autopsy, for example.[43] Although the presence of Factor V Leiden is an important risk factor for pulmonary embolism or deep venous thrombosis during pregnancy or oral contraceptive use,[44] there are as yet not generally accepted guidelines for use of Factor V status in clinical management.

A variety of laboratory methods are available for detection of Factor V Leiden mutation, including allele-specific PC1,[45,46] PCR/RFLP (the most commonly employed approach),[47] heteroduplex analysis,[48] single-stranded conformational polymorphism,[49] and denaturing gradient gel electrophoresis.[50] PCR/RFLP is based upon the fact that the point mutation in the Factor V gene lies within an Mnl 1 restriction enzyme recognition sequence, thus the amplified mutant allele(s) after enzyme digestion generate a different pattern of bands in an ethidium bromide-stained agarose or polyacrylamide gel. All methods are capable of detecting both mono- and biallelic mutations, so the choice of method is largely dependent upon convenience to the laboratory.

Although commercially available controls are not available, the allele frequency in the Caucasian population is sufficiently high to allow identification of a heterozygous control during assay implementation and validation.

## 14.3 Prothrombin Mutation

The 20210 G→A mutation in the prothrombin gene, with an allele frequency of nearly 2% in the Caucasian population, is a common genetic risk factor for deep venous thrombosis,[51] myocardial infarction,[52,53] and cerebral thrombosis.[54] Mutation in a single allele is associated with an approximately fourfold increase in the risk for deep venous thrombosis.[55] A variety of test formats have been proposed,

[f] Testing for these and many other diseases is conducted by fewer than 15 laboratories in the U.S.

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including heteroduplex analysis (as a multiplex test together with Factor V Leiden),[56] denaturing gel gradient electrophoresis,[57] single-strand conformational polymorphism,[57] allele-specific PCR,[58] and PCR/RFLP methods.[59,60] Although tissue from any site may be used for these analyses, buccal scrapings and blood are most commonly employed. The choice of laboratory techniques depends primarily upon the convenience and preferences of laboratory staff, since each of these tests, properly performed, is capable of unambiguously identifying the mutation and determining whether it is present on both alleles, or only one.

Commercial controls are not available. In general, laboratories are advised to seek initial control specimens from another laboratory already performing this assay. The allele frequency is sufficiently high to allow identification of additional heterozygous controls during the course of test validation and routine clinical testing.

### 14.4 Hemophilia A

Hemophilia A is an X-linked, recessive, bleeding disorder characterized by variable degrees of hemorrhage into joints and muscles, easy bruising, and prolonged bleeding from wounds. Approximately one individual in five to ten thousand is affected. The disease is caused by a deficiency in the activity of coagulation factor VIII. There is a wide spectrum of mutations, including point mutations (46%), inversions (42%), and deletions (8%).[61]

Laboratory identification of hemophilia A mutations may be accomplished by denaturing gradient gel electrophoresis;[62] this technique is capable of identifying nearly all mutations in patients with mild to moderate hemophilia, but is capable of identifying only about half of the mutations occurring in patients with severe hemophilia.[63,64] By using a set of 45 primer pairs, 99% of the region in which these mutations are found can be amplified and characterized by DGGE.[64]

Because of the great complexity of hemophilia mutation assays, it is prudent for laboratories not to perform these assays unless they expect to receive a relatively large number of samples.

## 15 RNA-based Assays

### 15.1 Introduction

Steady-state messenger RNA (mRNA) levels are one of the many mechanisms controlling gene expression. The steady state mRNA level is the result of the balance between mRNA production and degradation. The generation of a mature mRNA involves the synthesis of a primary transcript and the intranuclear processing of that primary transcript. The intranuclear processing of the primary transcript presents both problems and opportunities for RNA-based diagnostic testing. Differential splicing with alternative exon usage in the mature mRNAs can make the detection of a specific transcript complex. The juxtaposition of exonic sequences, which are far apart in genomic DNA, often makes mRNA a more readily amplifiable target. The size difference in the products generated by the same primers can be used to distinguish products generated from RNA from those of genomic DNA.

In molecular hematology the diagnostic amplification of mRNA targets is of greatest use in the detection of leukemia and lymphoma-associated chromosomal translocations.[65] Some of the more common translocations tested for, in this way, are shown in Table 9. The majority of these translocations result in the fusion of two genes to generate chimeric genes which encode a ”fusion” mRNA. The function of the oncogenic fusion proteins is derived from the reassortment of functional domains encoded by the two germ line genes. Typically the chimeric gene product encoded by only one of the two derivative chromosomes is required for the neoplastic phenotype. Molecular detection of the chimeric RNA is therefore an excellent marker of the disease. At a genomic level the chromosomal breakpoints are typically in introns and may occur anywhere within a several-kilobase region. These regions are often too

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large to be spanned by conventional amplification protocols, making molecular detection of the chimeric gene impractical. However, the structural requirements for oncogenesis lead to a focusing of the points of fusion within the chimeric mRNAs. This leads to a predictable pattern of fusion mRNAs generated from the derivative chromosomes and relatively small, easily amplified fusion and DNA sequences. Often the exons at the region of the fusion are small and not vital for leukemogenesis. These breakpoint region exons are alternatively located on either of the derivative chromosomes resulting in slight variation in the fusion point from patient to patient with the same translocation syndrome. In addition to the variability in genomic breakpoint positions, alternative splicing may include or exclude certain exons in the fusion mRNA. These factors can lead to a complex set of alternative amplification product, all of which are diagnostic of the translocation. Therefore, the relative importance of both derivatives, the patient-to-patient variability in fusion points, and possible alternative splicing patterns are important considerations in designing RNA amplification-based molecular diagnostic tests.

Important clinical applications using refinements of fusion transcript detection have been described. One of these employs multiplex PCR to use the same tube to screen for a number of common leukemia-associated translocations.[66] A quantitative analysis of the fusion transcript can be of greater utility than qualitative results. For example, positivity for the BCR-ABL fusion transcript using a sensitive RT-PCR assay after allogenic bone marrow transplantation is not incompatible with long-term remission. Increasing level of fusion transcript may reflect increased transcriptional activity or increasing tumor burden and has been shown to predict clinical relapse.[67]

In addition to the detection of chimeric mRNAs produced by chromosomal translocations, RNA-based assays can be used to identify gene products not typically expressed in hematopoietic cells such as *WT1* mRNA in the bone marrow[68] or the myeloperoxidase mRNA in peripheral blood.[69] Messenger RNA-based protocols can also offer a more economical approach to scanning for point mutations which can occur throughout a gene's coding sequence.

In all of these applications there are fundamental principles which can be applied to using mRNA as a target for amplification-based diagnostics in hematology. The most widely used amplification technology is the polymerase chain reaction (PCR), and it will be emphasized here. However, many of the general principles stated are applicable to other target and signal amplification technologies.

## 15.2 Test Design and Validation

The process by which an RNA-based test is developed and validated within the lab should be applied similarly to all new RNA-based tests. The design and validation of any RNA-based test should include:

- Thorough review of the literature with special attention to the normal patterns of gene expression and splicing of the transcript and any genes from which it is derived. Care should be taken in the design of the test so that the protocol (e.g., RNA yield and quality, primers, detection method) will detect the target transcript with appropriate sensitivity for the clinical setting in which it will be employed.

- Analytic and laboratory correlation studies to:

  — characterize the transcript to be detected;
  — establish the performance properties of the test to ensure the test's ability to provide consistent and reliable results;
  — determine aspects of the procedure which should be regulated to maintain test performance; and
  — define the relevant technical and clinical limitations of the test.

One of the most important applications of amplification-based RNA testing in molecular hematology is the detection of fusion transcripts encoded by leukemia-specific translocations. The most common

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technique employed is RT-PCR. In the design of such a test the choice of primers is critical for its clinical performance. For example, in t (9;22)/Philadelphia chromosome-positive leukemias, different primer pairs are used to distinguish between the transcript which encodes the p210 chimeric protein and that which encodes the p190 chimeric gene product. For both, the downstream primer is often placed in ABL exon 2. However, this results in a failure to detect BCR-ABL fusions which have a breakpoint distal to ABL exon 2.[70] In addition, other rare BCR-ABL fusions have alternative junctions and require different primers for detection.[71] These variants are rare, and a laboratory may choose to design the test so that it will not detect these variants.

Any method of detection can be complicated by alternative breakpoints and splicing patterns. For example, 10% of t(15;17) PML-RARa breakpoints occur within a PML exon and result in chimeric transcripts of slightly different sequences than if a PML intronic breakpoint occurs. These transcripts may result in failure to hybridize to probes used in Southern analysis or hybrid capture. The t(15;17) also can produce a number of alternatively processed chimeric transcripts resulting in a complex pattern of RT-PCR products on gel electrophoresis.[72]

In the analysis of minimal residual disease, the source of a specimen and its handling can be critical to the generation of accurate results. Any test producing quantitative results must provide information in the clinically relevant range of detection. These complexities must be fully appreciated prior to the implementation of testing.

In general the laboratory validation analysis in this process should include:

- A verification of the laboratory's ability to isolate high-quality RNA from the tissue of interest from *several* individuals using material whose handling is reflective of the specimen procurement system which will be used in actual testing. For example, in an RT-PCR test for a translocation fusion transcript, RNA should be prepared from *several* patients known to have the translocation. Well-characterized cell lines can provide a useful source of such material.

- A verification of the specificity of the amplification and detection method to be employed using the target in *several specimens* with and without the target present. For example, in an RT-PCR test for a fusion transcript, the transcript should be detectable in patients known to have the translocation but not in normal controls or patients in whom the available clinical and laboratory data makes the presence of the fusion transcript extremely unlikely. This work can be facilitated by the parallel analysis of the same material in an outside lab with an established protocol in this testing area.

- A verification of the sensitivity of the test appropriate to the clinical setting in which it will be used using material from *several specimens*. For example, for an RT-PCR test to monitor minimal residual disease, the test should perform with adequate sensitivity in separate analyses using cells or RNA from individuals known to contain the translocation diluted into appropriate normal cells or RNA.

- A verification of the precision of the test upon repetition of the sensitivity and specificity analyses with the same material on multiple runs.

- If the test is to produce a quantitative result, the precision of the assay across the full range of clinically relevant quantitation should be evaluated using multiple, independent RNA sources.

- If the assay is to use a multiplex format, the conditions must be optimized for that format, and validation of the individual components is not a substitute for validation in the multiplex format.

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## 15.3 Reagents

The protocol generated for an RNA-based test must include a control of the reagents employed in this testing. Lot-to-lot variability in the quality of reagents must be monitored and documented. To assure a new lot's quality, it should not be used in testing until after its quality is assured by using it in parallel with an existing lot. In RNA-based testing an important concern is contamination of any new reagents (even from the same lot) with ribonucleases (RNases). Parallel testing of new reagents used in parts of procedure where RNA is employed must be performed to verify the absence of RNase activity. Commercially available systems for the detection of RNase activity may be useful, but their use is not a substitute for verification of the absence of RNase by the assessment of RNA quality. In target amplification protocols there is also the danger of contamination of new reagents with target nucleic acid template. This contaminant can be DNA or RNA. To prevent the introduction of such contaminants into a testing system, new reagents should be demonstrated not to produce product in several blank amplification reactions prior to using in testing.

## 15.4 Specimens

Acceptable sources of material must be defined by the testing protocol. Isolation of amplifiable RNA has been described from a variety of sources including blood, marrow archival glass slide smears,[73] and paraffin-embedded tissue sections.[74] The conditions and time constraints of specimen handling, storage, and shipment to the lab must be defined in the testing protocol. Certain tissues and cell lineages are richer in RNases, and therefore may be more sensitive to specimen handling. False negatives can result from a delay in processing. This is especially of concern when testing for minimal residual disease where the neoplastic cell type may have less stable RNA than normal cell types present in the same specimen.

Because of the ubiquity of RNases, special care in specimen handling is required. From the beginning of the process, steps should be undertaken not to introduce RNases into the specimen. These steps should include strict adherence to the use of gloves when handling specimen collection supplies, and the segregation of these supplies should be considered. Heparin should not be used as an anticoagulant, because it is an inhibitor of many enzymes used in target amplification; EDTA or acid citrate dextrose are preferred anticoagulants. Specimen temperature is an important consideration. Bone marrow, blood, and other fluids containing erythrocytes should not be frozen, because red blood cells lyse upon thawing, releasing heme which inhibits many enzymes used in target amplification. Instead specimens containing erythrocytes should be transported to the lab on wet ice. Solid tissues should be snap frozen. RNA in frozen tissue can be stable for over one year. The specimen shipment process should allow for the extraction of RNA within four hours of collection if the specimen cannot be frozen.[75] A lab may decide to undertake testing on material that does not meet the lab's usual requirements for specimen handling. Any deviation from protocol should be noted in the final report, because such deviations may cause false-negative results; the readers of the report should be warned of this danger.

## 15.5 RNA Isolation

Clinical testing in which the molecular target is RNA generally gives the best results when the RNA is purified. Several protocols and solvent systems are available for the isolation and purification of RNA. As with the isolation of DNA, numerous methods have been reported in the literature, and commercial products are available from a number of different companies. As with all procedures, the laboratory should carefully follow the established protocol and incorporate established controls to verify proper performance for each extraction.

RNA is very sensitive to degradation by RNases which may be of exogenous or endogenous origin. Common sources of exogenous RNAse contamination include glassware, plasticware, laboratory solutions, autoclaves, and laboratory personnel. Therefore, strict adherence to hand-gloving policies, including frequent glove changes, is necessary when working with RNA. Extra care is necessary to ensure

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that all reagents and supplies used in the extraction and storage of RNA are properly treated to destroy or inhibit RNases. Aqueous solutions can be treated with diethylpyrocarbonate (DEPC). However, DEPC can inactivate other enzymes used in later stages of the test, and it must be removed by autoclaving for at least 45 minutes. Glassware must be baked at 200 °C for four hours to eliminate RNAse contamination. A variety of commercial preparations are available that inhibit RNAse activity; however these can also be a source of RNAse contamination, because on prolonged storage RNAse may prove more durable than the inhibitor. The RNA-based testing protocol should include a systematic approach to assure that exogenous RNAse contamination is minimized.

In addition to exogenous sources of RNAse, the protocol must deal with RNAse from the specimen itself. The level of RNAse varies with different tissues and cell lineages.[76] In complex mixtures of cell types such as the bone marrow, this can lead to false negatives from the cells of interest even if high-quality RNA was isolated from other cells in the preparation. Removal of eosinophils from hematologic specimens may improve assay results by removing the major source of ribonuclease contamination.[77,78] The endogenous RNases are inactivated by denaturation in most RNA preparation protocols. Some protocols employ sodium dodecyl sulfate (SDS) for this denaturation. It is important to note that SDS can inhibit enzymes used for target amplification such as *Taq* polymerase. The type of RNA made will influence the sensitivity and complexity of the assay. Only 2 to 3% of total cellular RNA is mRNA. The purification of mRNA can be accomplished by selection for RNA with 3' poly-(A) tails which will increase the sensitivity of many amplification protocols. However, poly-$(A)^+$ selection is usually not necessary in clinical testing. Total cellular RNA (in contrast to cytoplasmic RNA) includes incompletely processed transcripts which can complicate the detection of any cDNA amplification products. The contamination of RNA preparation with DNA can also complicate detection and may be eliminated with RNAse-free DNAse.

It will often be useful to assess RNA quality and concentration prior to amplification. This is important because amplification controls, which should also be used to assure the quality of RNA template, do not produce results until the end of the testing protocol. The identification of RNA quality problems prior to amplification will result in more efficient troubleshooting and better patient management. If a specimen produces only degraded RNA, another specimen may be requested and obtained in a timely fashion. Gel electrophoresis offers a quick, straightforward method to assess the intactness of total RNA. The presence of sharp 18S and 28S bands generally indicates quality RNA, and these gels can also be used to estimate RNA concentration. RNA concentration is typically determined by spectrophotometry. Nucleic acids absorb maximally at 260 nm and proteins maximally at 280 nm. The RNA concentration can be determined based on the fact that each O.D. unit at 260 nm is equivalent to 40 µg/mL of single-stranded RNA. An O.D. 260/280 ratio provides a qualitative measurement of the level of RNA with respect to the amount of contaminating protein that exists in the sample. Ratios of 1.9 to 2.1 indicate high levels of RNA purity. Purity may be improved by re-extraction and precipitation when ratios obtained are below 1.6. Human leukocytes generally yield 10 to 40 µg of total RNA per $10^6$ cells.

Isolated RNA should be stored in tightly capped containers. Long-term storage should be carried out at -70 °C or in liquid nitrogen or ethanol to prevent degradation. Stability of the sample can be maintained in aqueous solution for several months by storing at -70 °C in tightly capped containers. Integrity of samples should be re-evaluated before use if stored for extended periods of time at any temperature.

## 15.6 Amplification

The most common amplification technology used for the detection of RNA in hematology is reverse transcription-polymerase chain reaction (RT-PCR). This section will cover that technology in detail and then discuss alternative amplification technologies.

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### 15.6.1 Reverse Transcription

Prior to the PCR amplification of a target RNA, it must be converted to a single-stranded DNA by a reverse transcriptase. There are three major considerations in the design of this step: the concentration of RNA to be used, the choice of primer, and the choice of the specific reverse transcriptase. The amount of RNA used in the reaction will determine the sensitivity of the assay in the minimal residual disease setting. It should be optimized and then specified in the protocol. In some cases the optimal amount of RNA may not be available. The test can still be performed, but this deviation from the standard protocol should be specified in the report. The primer used for generation of cDNA may be:

- Oligo-dT;
- Random hexamers; or
- Gene specific sequence.

The use of oligo-dT requires the target sequence to be polyadenylated. Random hexamer often gives the greatest sensitivity and flexibility. Gene-specific primers are often required in single-tube RT-PCR protocols which include both reverse transcriptase and DNA polymerase activities in the same reaction. Reverse transcriptases that are RNAse H negative give a higher percentage of full-length cDNAs. This can be important if the target amplification product is long or if the target is near the 5' end of the gene. RNAse inhibitors can be included in the RT reaction to help maintain the integrity of the template. RNA secondary structure can reduce the efficiency of the reverse transcription step, and the conditions of this step need to be optimized for maximum sensitivity. The RT step can also be the source of contamination; therefore aerosol-filtered tips or positive displacement pipettes should be used when transferring RNA or the product of the RT reactions.

### 15.6.2 Polymerase Chain Reaction (PCR)

The polymerase chain reaction offers the potential of exponential amplification of target DNA sequences. This offers both the potentials of great sensitivity and contamination. The general considerations regarding the design and execution of PCR discussed above apply to RT-PCR. The amount of cDNA used in the reaction will determine the sensitivity of the assay in the minimal residual disease setting. It should be optimized and then specified in the protocol. Considerations specific to RT-PCR will be discussed in this section. In general, the primers should be chosen to amplify across the junction of two exons. This will result in different products being generated from RNA and genomic DNA. Consideration must also be given to alternative splicing and disease-associated DNA rearrangements which may exclude the primer sequences from the mature mRNA and reduce a test's ability to detect all possible target transcripts. In most PCR protocols the presence of RNA will not significantly reduce amplification efficiency, but it may complicate detection procedures. Reverse transcriptases, when still active, can inhibit the DNA polymerase activity of some thermostable polymerases.[79] If a multiplex PCR protocol is to be employed, it is likely to reduce the sensitivity of detection of any of the individual transcripts. Multiplex PCR often requires additional thermostable polymerase per reaction volume when compared to single-target amplification.

The detection of PCR products should include a sequence-based verification of the identity of the PCR product. Common valid approaches employed include oligonucleotide probes, nested PCR, or restriction digestion. Oligonucleotide probes may be used in liquid or Southern-blot hybridizations or for solid-phase capture of the PCR products. These probes should be specific for sequences internal to the PCR primers and should be designed to detect all significant alternative splicing patterns.

Nested PCR offers additional sensitivity as well as the added specificity derived from probing both ends of the PCR product. However, nested PCR has the potential for serious problems with contamination from other tubes and existing PCR products. In nested PCR, tubes containing first-round PCR product should be opened one at a time to prevent tube-to-tube contamination, and each step of RT-PCR must be

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controlled with template blanks. The use of photochemical reagents such as psoralens or enzymatic contamination controls such as uracil-N-glycosylase cannot be employed to control cross-contamination between first- and second-round nested-PCR reactions.

### 15.6.3 Quality Controls for RT-PCR Tests

RT-PCR-based tests need to include positive-, sensitivity-, negative-, amplifiability-, and reagent-contamination controls in each run. In RT-PCR protocols where the primers employed result in products which do not distinguish between products of genomic DNA and RNA origin, control reactions without reverse transcriptase must be included for each template. Positive controls can be from cell lines or patient material containing the transcript to be detected. RNA from positive controls should be used at concentrations which will not produce excessive amounts of PCR products, because these can be the source of contamination. Each run should include a clinically relevant sensitivity control to verify the lower limit of detection of target. This is particularly important when testing for minimal residual disease. For example, the typical RT-PCR for a translocation-associated fusion transcript will be able to detect one cell expressing the fusion transcript when diluted into 10,000 to 100,000 cells. The positive and the sensitivity control may be the same, but the assessment of ability to detect different concentrations of target has merit. A negative control containing high-complexity cellular RNA in the absence of the target transcript should be included in each run.

Amplifiability controls address both the quality of the RNA and the presence of polymerase inhibitors. Typically, a ubiquitously expressed transcript is used as a target for the amplifiability control reaction. This control assesses both RNA quality and the RT-PCR reagents used. It can be multiplexed with the reaction for the target transcript, but this may result in reduced sensitivity for the target. β-actin is often used as this control, but it has two important drawbacks: its level may vary with different physiologic conditions, and is often much higher than the mRNA detected by the test; in addition, untranscribed β-actin pseudogenes may be amplified[74] leading to incorrect conclusions about RNA quality. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine phosphoribosyltransferase (HPRT), and the c-ABL genes offer better alternatives for this control. Failure to amplify this control target indicates the RNA is of poor quality, there is a polymerase inhibitor present, or RT-PCR has failed in that tube. Alternatively, a synthetic internal control can be employed. The presence of a polymerase inhibitor or RNAase can be further controlled for by spiking patient material with low levels of known target sequence. Multiplexing the amplification of a universally expressed target with the amplification of the test target can control for individual tube failures. Individual tube failures may result from poor mixing of reaction components or thermocycler block nonuniformities. It should be noted, however, that low levels of a target sequence may not be detected despite intact amplification in a given tube. This is because of the stochastic nature of amplification in early cycles of PCR from rare templates. To guard against false negatives at the limits of detection and single-tube reaction failures, all test specimens should be assayed at least in duplicate. Template blank reactions for all steps of RT-PCR should be included in each run and should be the last tubes of a run. Such template-negative blanks assure the reagents are not contaminated but do not rule out tube-to-tube contamination within a run.

### 15.6.4 Quantitative Techniques

The greatest clinical utility may be derived from a quantitation of a transcript rather than its mere qualitative detection. Target amplification techniques have been developed which can do this, but because these techniques often involve exponential amplifications, they require detailed verification and careful use of controls in each run. Signal amplification techniques have also been developed. Often their use in transcript quantitation is more straightforward, but they lack the sensitivity of target amplification strategies.

The most commonly used quantitative methods employ PCR.[80] The replication at each step of a PCR reaction is not perfectly efficient, and the degree of efficiency varies with each primer/template

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combination. At higher cycles amplification proceeds with lower efficiency and is no longer exponential, as components become limiting and the products compete with targets for primers during annealing. Eventually a plateau effect develops so that the amount of product produced no longer reflects the amount of template initially present. Therefore, in many protocols quantitation requires maintaining PCR in the exponential phase.

The simplest method of quantitation is the measurement of the PCR product produced and its comparison to an external standard such as a reference curve. Because of tube-to-tube variability and differences in efficiency, this method may lead to inaccurate results, unless linear regression analysis on the amount of product is done at multiple time points in replicate tubes throughout a reaction. The recent development of thermocyclers with the capability to continuously measure product levels will facilitate the use of this method of quantitation with greater accuracy. This approach has been referred to as "real-time PCR" and will be discussed further below.[81]

Limiting dilution is a method of quantitation which depends on loss of a positive signal. It can produce satisfactory results but requires that the limit of detection is reproducible and that multiple reactions be performed at each dilution so that Poisson statistics can be applied to the data. In the absence of this, statistical analysis errors of two orders of magnitude can occur. Coamplification of a target and a control transcript which is present at steady-state levels can be used to generate a relative quantitation. Quantitative analysis is often performed in the setting of analysis of minimal residual neoplasm. The coamplification method is difficult to use in this setting because the control gene may be expressed at different levels in neoplastic and background normal tissues.

The most commonly used quantitative RT-PCR method is competitive PCR. In this method a control template which can be amplified by the same primers is spiked into the PCR reaction, and the two templates compete for primers. Serial dilutions of competitor are coamplified with the target sequence (or vice versa). In competitive PCR, quantitation is based on the ratio of products generated from the control and target templates. The control and target must be amplified with equal efficiency, by the same primer pair. The control and target templates may be differentiated by the size of the products generated or differences in internal sequence. The control template may be either DNA or RNA, but the use of RNA presents problems inherent to RNA's instability. If probes are used to distinguish products generated from the target and competitor, the efficiency of hybridization must be controlled for. The parallel quantitation of a housekeeping gene by competitive RT-PCR can be used to control for RNA quality.

Special considerations must be addressed when real-time PCR is used. Quantitation in this method is typically based upon the first cycle at which specific product is detected above background signal. This cycle is referred to as the "threshold cycle ($C_t$)." External standard curves of the $C_t$ versus known concentrations of starting material are employed for quantitation of unknowns. There is a log-linear relationship between $C_t$ and starting concentration. Specific PCR product may be detected by fluorescent dyes specific for double-stranded DNA (such as SYBR® green) or with oligonucleotide probes whose fluorescence is dependent upon the presence of specific product. When the double-stranded, DNA-specific dye approach is used, the PCR reaction must be optimized so that no nonspecific product is generated. Over a range of clinical specimens, this may be a very difficult requirement to meet, and this approach runs the risk of false-positive results. The probe approach (such as the homogeneous PCR technology) typically employs fluorescent energy transfer and is less subject to false positives. Both approaches require that good signal-to-noise ratios be demonstrated across a range of initial concentrations and in a variety of clinically relevant specimens prior to the implementation of testing.

Any quantitative technique should be validated to determine the clinically relevant linear range. Extreme sensitivity may not be an advantage. Sensitivity, specificity, and, especially for quantitative techniques, precision must be demonstrated on multiple specimens over a range of initial template concentrations. Precision as expressed by coefficient of variation (%CV) should be calculated both within and between

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runs using the same specimen; and acceptable precision should be defined prior to the implementation of testing and routinely monitored during testing.

## 15.7 Reporting

Reports should include statements of the methodology used, results of the test, sensitivity of the test, and the limitations of test. The statement of the methodology used should define the method of amplification and the sequences targeted by that amplification. Positive results should include any patient-specific features of the positivity, such as band sizes or breakpoint positions. If the results are quantitative, the range of possible error and denominator must be clearly defined. If the result is negative, a statement of the quality control steps taken to exclude a false negative should be included. The sensitivity of the test should be verified on each run and stated in the report. The report should also define the limitations of the test such as those imposed by primer sequences in an RT-PCR test. Any significant deviation from standard testing protocol should also be specified in the report.

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**Table 9. Common Translocations Tested for Diagnostic Amplification of mRNA Targets**

| Translocation | Disease | Genes Involved |
|---|---|---|
| t(9;22)(q34; q11)/ p210 | CML | *ABL* (9q34)<br>*BCR* (22q11) |
| t(9;22)(q34; q11)/ p190 | ALL | *ABL* (9q34)<br>*BCR* (22q11) |
| t(1;19)(q23; p13.3) | pre-B-ALL | *PBX1* (1q23)<br>*E2A* (19p13.3) |
| t(17;19)(q22;p13) | pro-B-ALL | *HLF* (17q22)<br>*E2A* (19p13) |
| t(15;17)(q21-q11-22) | APL | *PML* (15q21)<br>*RARA* (17q21) |
| t(4;11)(q21; q23) | ALL/pre-B-ALL<br>ANLL | *MLL* (11q23)<br>*AF4* (4q21) |
| t(9;11)(q21;23) | ALL/pre-B-ALL<br>ANLL | *MLL* (11q23)<br>*AF9/MLLT3* (9p22) |
| t(11;19)(q23;p13.3) | Pre-B-ALL/T-ALL<br>ANLL | *MLL* (11q23)<br>*ENL* (19p13) |
| t(11;19)(q23;p13.1) | ANLL | *MLL* (11q23)<br>*ELL* (19p13.1) |
| t(8;16)(p11;p13) | ANLL | *MOZ* (8p11)<br>*CBP* (16p13) |
| t(8;21)(q22;q22) | AML | *AML1/CBFa* (21q22)<br>*ETO/MTG8* (8q22) |
| t(6;9)(p23;q34) | AML | *DEK* (6p23)<br>*CAN* (9q34) |
| inv(16)(p13;q22) | AML | *Myosin MYH11* (16p13)<br>*CB-* (16q22) |
| t(5;12)(q33;p13) | CMML | *PDGF-B* (5q33)<br>*TEL* (12p13) |
| t(2;5)(2p23;q35) | T-cell lymphoma | *NPM* (5q35)<br>*ALK* (2p23) |
| t(12;21)(p13;21q22) | ALL | *TEL*(12p13)<br>*AML1*(21q22) |

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## Additional Reference

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## Appendix. Figures

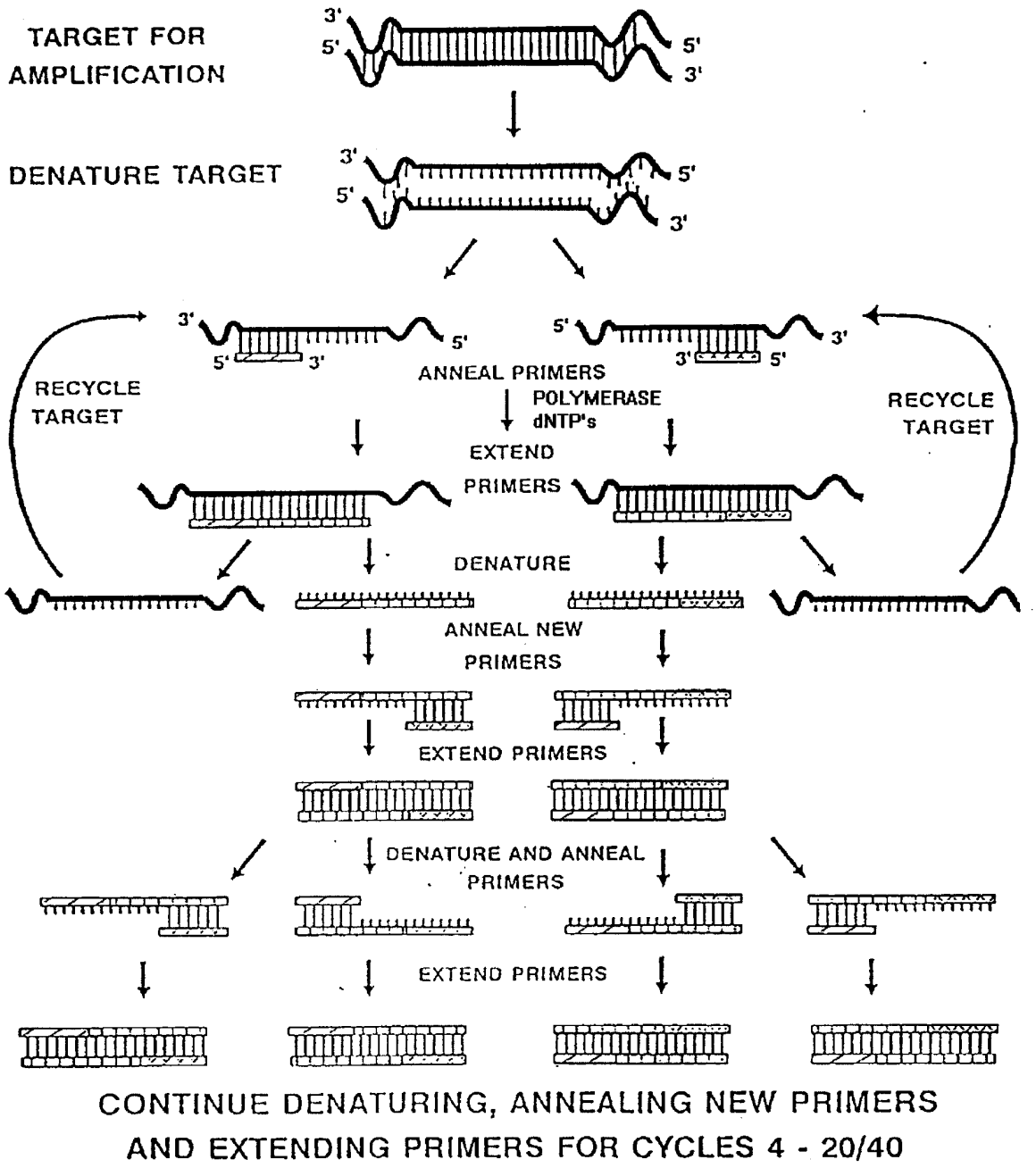


**Figure A1. PCR Amplification.** Reprinted from Wolcott MJ, Department of Microbiology, Iowa State University. DNA-based methods for the detection of foodborne pathogens. *J Food Protect.* 1991;54;5:387-401. Reprinted with permission from the *Journal of Food Protection.* Copyright held by the International Association for Food Protection, Des Moines, Iowa, USA.

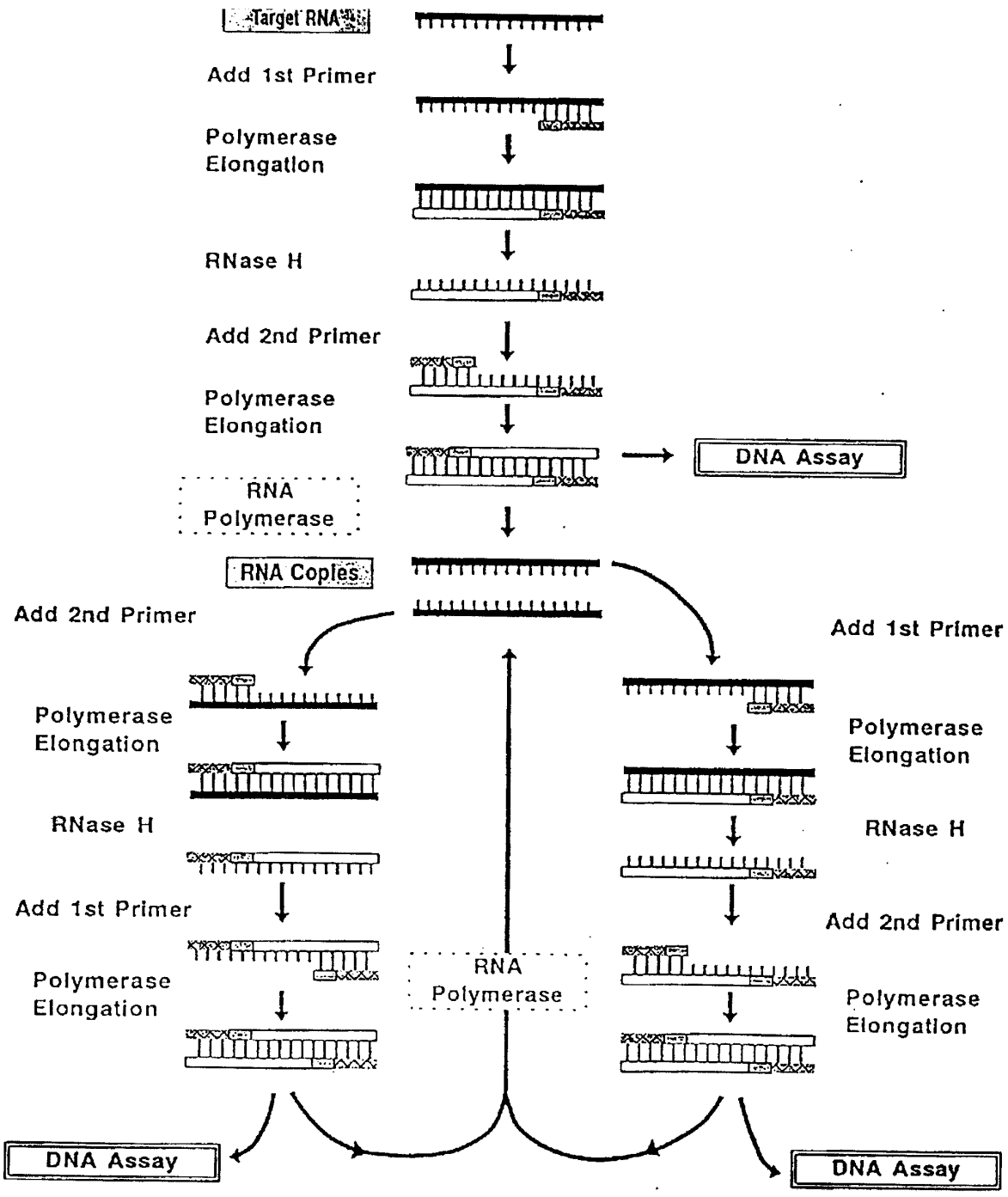


**Figure A2. 3SR Amplification.** From Wolcott MJ. Advances in nucleic acid-based detection methods. *Clin Microbiol Rev.* 1992;5:370-386. Reprinted with permission from American Society for Microbiology and Dr. MJ Wolcott.

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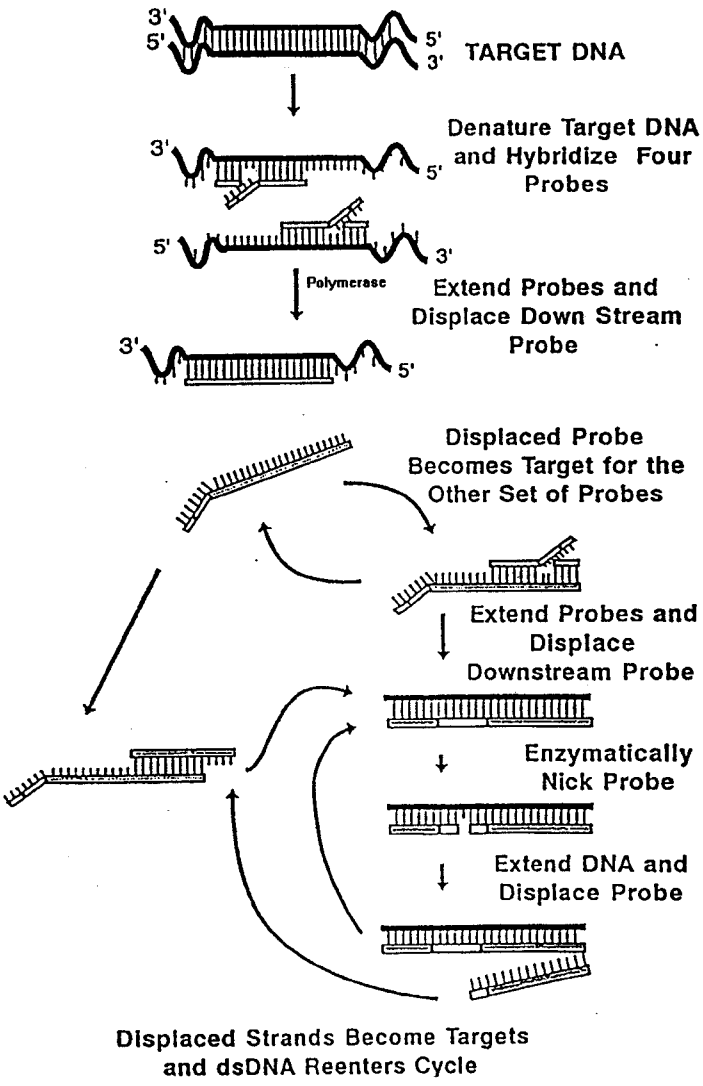


**Figure A3. Strand Displacement Amplification (SDA).** From Wolcott MJ. Advances in nucleic acid-based detection methods. *Clin Microbiol Rev.* 1992;5:370-386. Reprinted with permission from American Society for Microbiology and Dr. MJ Wolcott.

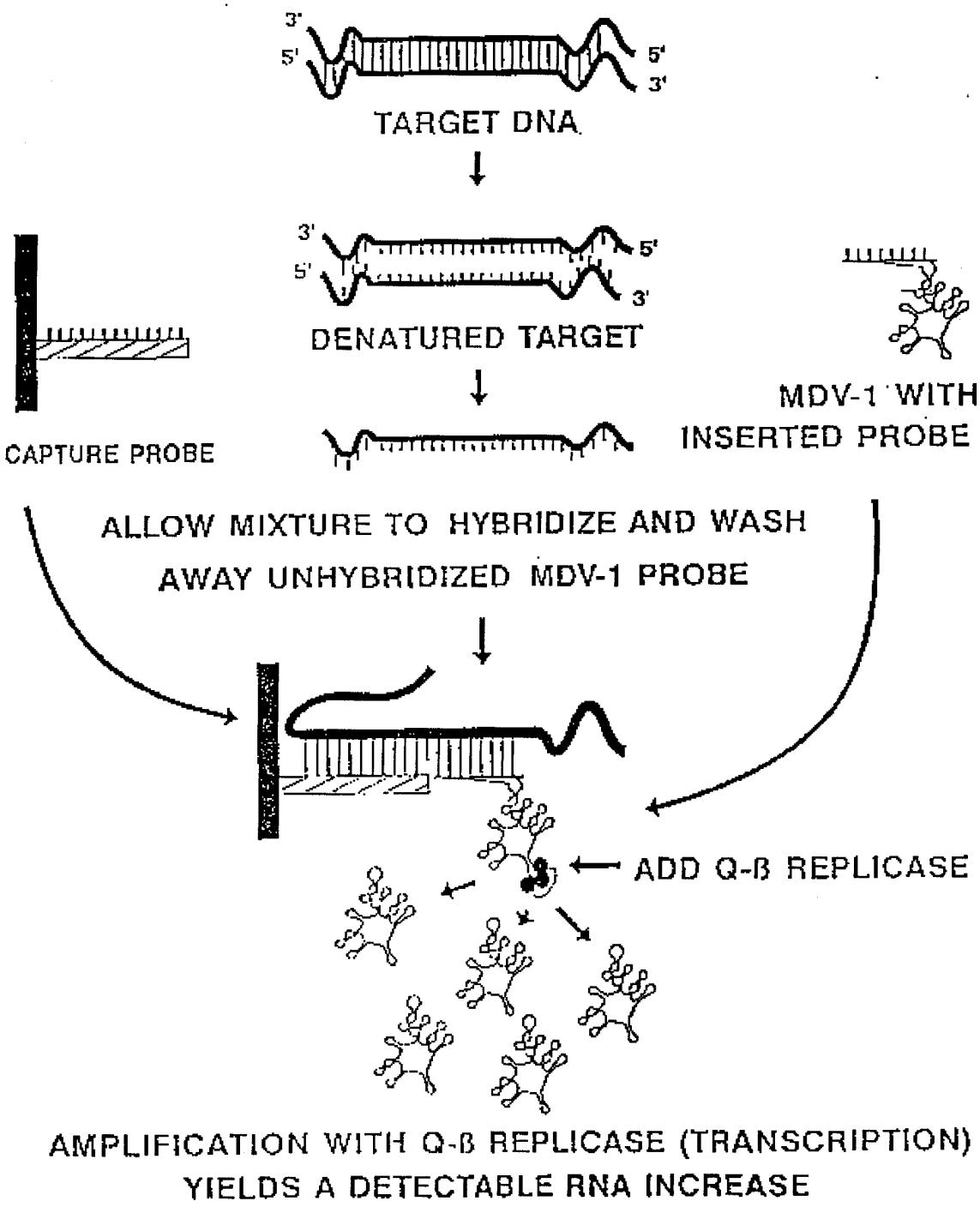


**Figure A4. Q-beta Replicase.** Reprinted from Wolcott MJ, Department of Microbiology, Iowa State University. DNA-based methods for the detection of foodborne pathogens. *J Food Protect.* 1991;54;5:387-401. Reprinted with permission from the *Journal of Food Protection.* Copyright held by the International Association for Food Protection, Des Moines, Iowa, USA.

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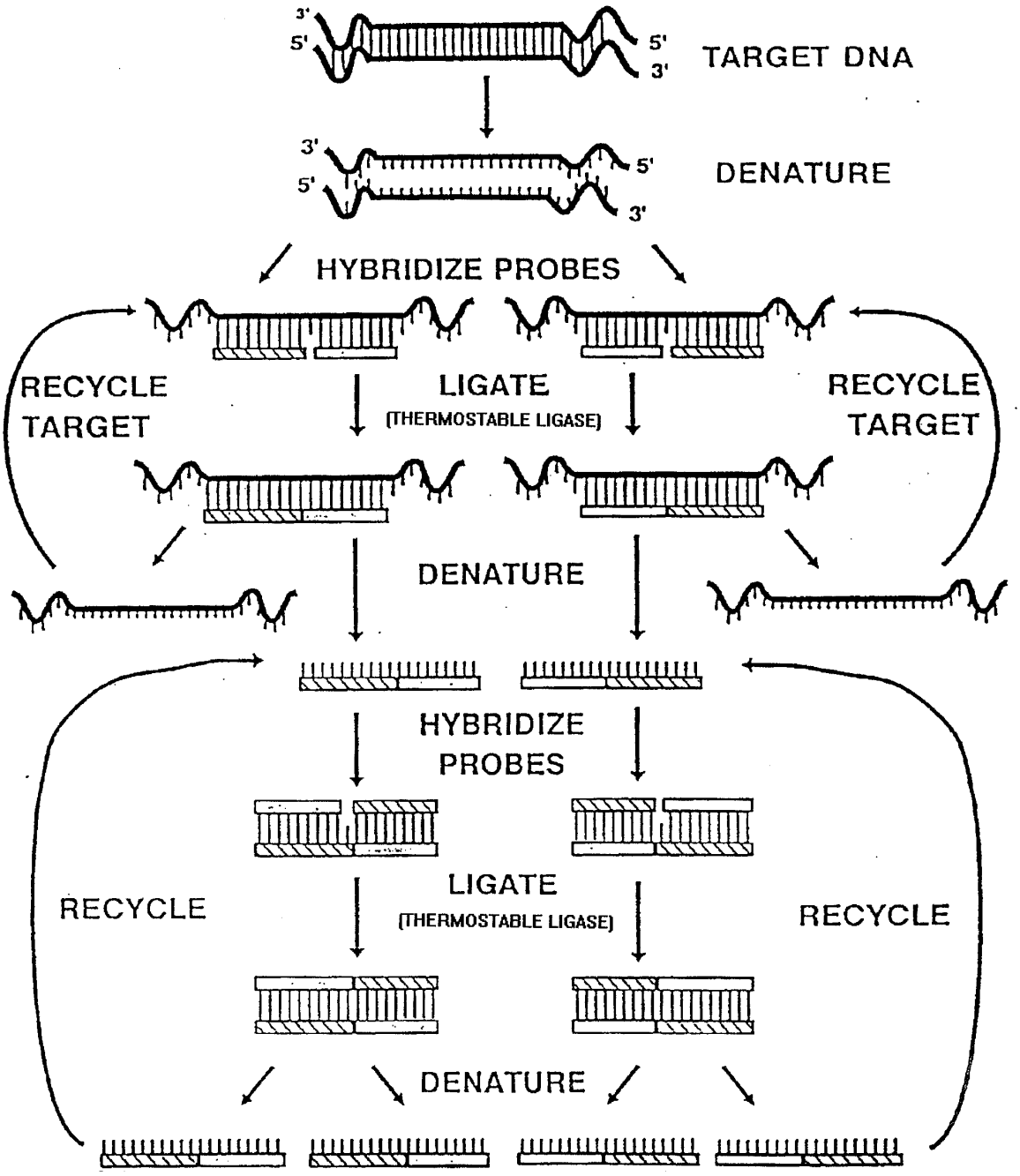


**Figure A5. Ligase Amplification of a Target by Use of Two Oligonucleotide Probes.** Reprinted from Wolcott MJ, Department of Microbiology, Iowa State University. DNA-based methods for the detection of foodborne pathogens. *J Food Protect.* 1991;54;5:387-401. Reprinted with permission from the *Journal of Food Protection.* Copyright held by the International Association for Food Protection, Des Moines, Iowa, USA.

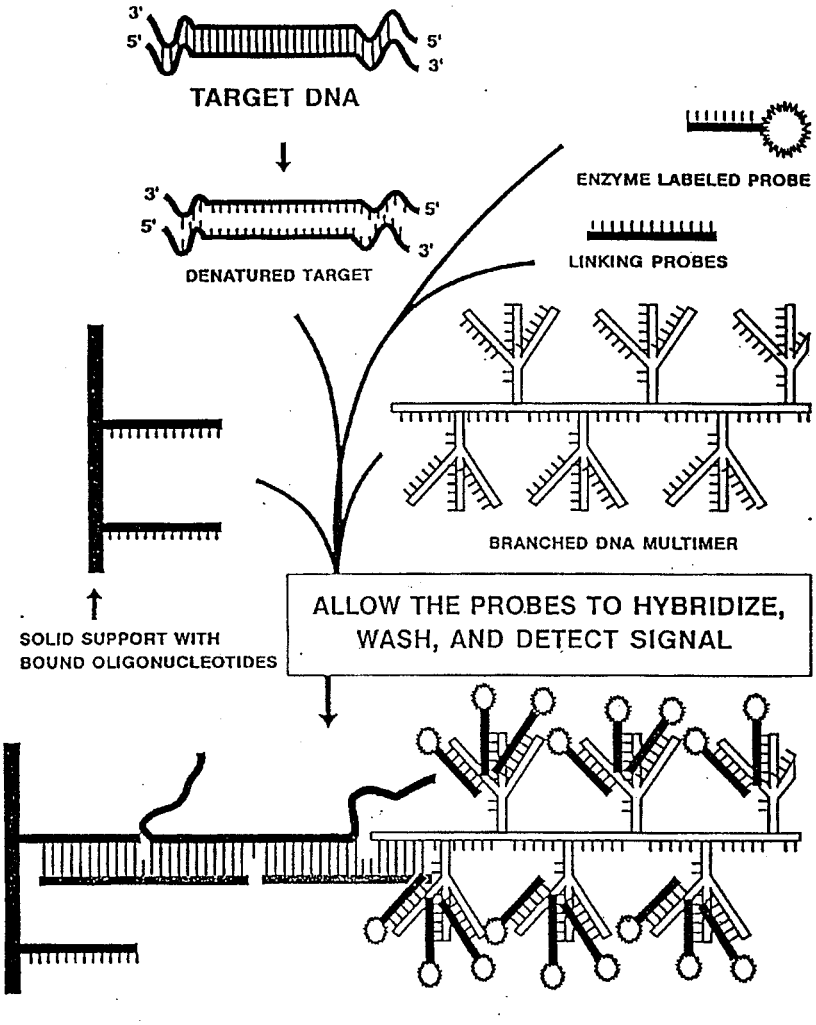


**Figure A6. Branched DNA Signal Amplification.** From Wolcott MJ. Advances in nucleic acid-based detection methods. *Clin Microbiol Rev.* 1992;5:370-386. Reprinted with permission from American Society for Microbiology and Dr. MJ Wolcott.

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## Summary of Comments and Subcommittee Responses

MM5-P: *Nucleic Acid Amplification Assays for Molecular Hematopathology; Proposed Guideline*

General

1. Where appropriate, cross-references to new area committee/subcommittee projects (e.g., MM7, MM8) should be provided.

- **The subcommittee concurs. References to related NCCLS molecular methods publications have been added, as appropriate.**

2. There are minor typographical errors that require correction.

- **Typographical errors have been corrected.**

Section 2

3. Scope of the document must be limited to chromosomal aberrations and mutations in cancer cells; germline mutations, abnormalities, polymorphisms or variants should not be included.

- **The subcommittee disagrees with this recommendation. These types of abnormalities all occur within the scope of practice of those performing molecular hematology assays.**

Section 3

4. There are too many definitions; not all are necessary for this guideline.

- **The Definitions section has been modified as suggested.**

Section 4.3

5. Any reference to identification of germline sequences corresponding to known causes of hematologic disease should be deleted.

- **The subcommittee disagrees with this recommendation. These types of abnormalities all occur within the scope of practice of those performing molecular hematology assays.**

Section 5

6. Specimen identification and accessioning, recordkeeping, record retention, and specimen retention should be consonant with NCCLS guidance for non-nucleic acid-based assays.

- **The subcommittee vigorously disagrees with this perspective. It is inconceivable that specimen retention should be the same for tumor DNA and for a blood specimen used to obtain a "stat" serum-sodium measurement. Specimen accessioning, record keeping, and record retention must be based upon the patient's best interest, which in turn, depends upon the nature of the clinical test.**

  **The recommendations contained herein have been aligned with NCCLS document MM2—*Immunoglobulin and T-Cell Receptor Gene Rearrangement Assays.***

Section 6

7. Specimen identification and accessioning, recordkeeping, record retention, and specimen retention should be consonant with NCCLS guidance for non-nucleic acid-based assays.

- **See the response to Comment 6 above.**

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Section 8

8. Quality control as defined in this section should be consonant with NCCLS guidance for non-nucleic acid-based assays, except for Section 8.3 specifying carry-over prevention.

- **The subcommittee appreciates and shares the commenter's perspective. Note, however, that there is no single NCCLS guidance document for quality control, but rather a number of guidance documents and standards that depend upon the nature of the test performed. There are unique aspects of nucleic acid-based diagnostics that give rise to unique recommendations for quality control procedures. It is particularly important that the NCCLS guideline is consonant with perspectives already encoded in accreditation guidelines (e.g., CAP Laboratory Accreditation Program guidelines and standards) and those guidelines and standards that have been promulgated by other expert groups (e.g., the Association for Molecular Pathology and the American College of Medical Genetics); otherwise, NCCLS guidelines lack credibility.**

Section 11.5

9. Test validation as defined in this section should be consonant with NCCLS guidance for non-nucleic acid-based assays.

- **The subcommittee appreciates and shares the commenter's perspective. Note, however, that there is no single NCCLS guidance document for test validation, but rather a number of guidance documents and standards that depend upon the nature of the test performed. There are unique aspects of nucleic acid-based diagnostics that give rise to unique recommendations for test validation.**

  **That nucleic acid-based assays are different has been clearly established by a number of advisory bodies and government entities, including (in the U.S.) the Secretary's Advisory Committee on Genetic Testing (SACGT) and the Clinical Laboratory Improvement Advisory Committee (CLIAC), and seems likely to be codified in the new CLIA regulations. Provision of additional recommendations, by NCCLS, for test validation of nucleic acid-based assays would be redundant and a duplication of effort.**

10. I am uncomfortable with the wording in the validation section, i.e., "several" specimens rather than a number. I recognize, however, that different assays will have different requirements and sample acquisition capabilities.

- **The members of the subcommittee were equally uncomfortable, but could not identify any reasonable approach to developing more concrete recommendations.**

Section 14

11. This section should be removed in its entirety.

- **See the response to Comment 5 above.**

Section 15

12. This section should be clearly restricted in scope to RNA-based assays from cancer cells.

- **See the response to Comment 5 above.**

References

13. The "Wolcott" reference on amplification schema is outdated.

- **Although the subcommittee believes most of the figures from Wolcott remain appropriate, Figure 7 is slightly "off the mark" for this document and has been deleted.**

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# Summary of Delegate Comments and Subcommittee Responses

MM5-A: *Nucleic Acid Amplification Assays for Molecular Hematopathology; Approved Guideline*

General

1. The document is much longer than necessary and most of the guideline is too general, i.e., not specific to Hematopathology. For example, could safely delete first 34 pages because material could apply to any of the NCCLS molecular diagnostic guidelines.

- **The subcommittee recognizes that much of the document is quite general, and that much of it may have applicability beyond the area of molecular hematology. Nevertheless, the subcommittee members considered it important that the document be "stand alone" in nature.**

2. Methodologies reviewed did not include real-time PCR with associated detection methods. This document will be quickly outdated.

- **The subcommittee has given some attention to "real time" PCR in Section 15.6.4, but recognizes that this topic may be worthy of a full document.**

  **Members of the subcommittee hope that the newer accelerated processes adopted by NCCLS will enable the preparation of more timely documents.**

3. There is no treatment of minimal residual disease validation.

- **The subcommittee members recognize that this is an important area, but believe that the general principles for documenting clinical validity are applicable to this.**

4. Could use discussion of how to interpret VDJ and T-cell clonality, e.g., numeric criteria for reporting clonality.

- **Although some authors have proposed numeric criteria for interpretation of clonality, particularly when using capillary electrophoresis methods, the subcommittee does not believe there is sufficient consensus on any particular standard to recommend specific numerical criteria.**

5. There is no discussion of bone marrow engraftment monitoring.

- **Members of the subcommittee recognize that this could legitimately be considered within the scope of this document, but did not believe they had sufficient expertise at the time of initial preparation to address it properly.**

Section 5.3

6. Paragraph 2 regarding cerebrospinal fluid is confusing. What does it mean when a laboratory has not received a sufficient number of specimens? Or does this sentence refer to insufficient past experience to obtain the expertise needed to provide quality laboratory service for these types of specimens?

- **The intent of this paragraph is to indicate one common reason why a laboratory director may choose not to accept certain types of specimens. The laboratory director is obligated to be certain that the laboratory has the technical competence to handle the specimen and perform the assay. Many laboratory directors believe that this requires a reasonably frequent performance of the assay in question, and currency of the laboratory staff in handling the type of sample that is submitted. The subcommittee does not intend to prescribe a method for conducting this assessment, but when considering currency a laboratory director may wish to consider both past experience and current specimen volume.**

  **The case of CSF specimen volume is only an example of a type of situation the laboratory director may encounter when deciding on criteria for specimen rejection. Each laboratory director must take into account the peculiar circumstances of his or her laboratory.**

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Section 8.1

7. The guideline states that the director can “OK” use of outdated reagents. This is in conflict with CAP and is against good laboratory practice as well as good manufacturing practices. The "comparative testing" to demonstrate acceptable results would have to be comparable to what the original manufacturer performed to set the original dating.

- **This is a difficult area. Within the United States of America, the current interpretation of the Clinical Laboratory Improvement Act of 1988 is that reagents may not be used beyond the expiration date set by the manufacturer. Nevertheless, subcommittee members believe there is evidence that in many cases these reagents could, in fact, be safely and effectively used after the manufacturer’s expiration date. Since NCCLS documents are global in scope, the subcommittee believes it inappropriate to rely on United States law or the College of American Pathologists in the creation of its guidelines. Nevertheless, the subcommittee also recognizes that a large number of users of these guidelines will be bound by United States law and practice, and that other nations and accrediting bodies may well be similar to those in the United States. For this reason, the statement has been revised to read:**

  **“Under most circumstances, reagents available through a manufacturer should not be used beyond the expiration date set by the manufacturer. In the case of rare reagents that are difficult to source, predetermined procedures and acceptance criteria that are predictive of the quality of the reagent should be documented and used to validate the efficacy of the reagent prior to use. Where permitted by law and the appropriate accrediting agencies, certain valuable reagents may be used beyond their expiration date, provided that comparative testing demonstrates continued acceptable results; these findings should also be recorded in a logbook and approved by the laboratory director. In the United States, this practice is not currently permitted.”**

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**NOTES**

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## 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:

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

MM5-A addresses the quality system essentials (QSEs) indicated by an "X." For a description of the other NCCLS documents listed in the grid, please refer to the Related NCCLS Publications section on the following page.

| Documents & Records | Organization | Personnel | Equipment | Purchasing & Inventory | Process Control | Information Management | Occurrence Management | Assessment | Process Improvement | Service & Satisfaction | Facilities & Safety |
|---|---|---|---|---|---|---|---|---|---|---|---|
| GP2-A4<br>MM7-P | | MM7-P | MM7-P | GP9-A | X<br>MM1-A<br>MM2-A2<br>MM3-A<br>MM4-A<br>MM7-P | | | MM7-P | MM4-A | | MM7-P |

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

### 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, GP26-A2 defines a clinical laboratory path of workflow which 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.

MM5-A addresses the clinical laboratory path of workflow steps indicated by an "X." For a description of the other NCCLS documents listed in the grid, please refer to the Related NCCLS Publications section on the following page.

| **Preanalytic** | | | | | **Analytic** | | **Postanalytic** | |
|---|---|---|---|---|---|---|---|---|
| Patient Assessment | Test Request | Specimen Collection | Specimen Transport | Specimen Receipt | Testing Review | Laboratory Interpretation | Results Report | Post-test Specimen Management |
| X<br>MM1-A<br>MM2-A2<br>MM7-P | X<br>MM1-A<br>MM2-A2<br>MM7-P | X<br>MM1-A<br>MM2-A2<br>MM3-A<br>MM4-A<br>MM7-P | X<br>MM1-A<br>MM2-A2<br>MM3-A<br>MM4-A<br>MM7-P | X<br>MM1-A<br>MM2-A2<br>MM4-A<br>MM3-A<br>MM7-P | X<br>MM1-A<br>MM2-A2<br>MM3-A<br>MM4-A<br>MM7-P | X<br>MM1-A<br>MM2-A2<br>MM3-A<br>MM7-P | X<br>MM1-A<br>MM2-A2<br>MM3-A<br>MM4-A<br>MM7-P | X<br>MM1-A<br>MM2-A2<br>MM4-A<br>MM7-P |

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

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## Related NCCLS Publications*

**GP2-A4** **Clinical Laboratory Technical Procedure Manuals; Approved Guideline – Fourth Edition (2002).** This document provides guidance on development, review, approval, management, and use of policy, process, and procedure documents in the laboratory testing community.

**GP9-A** **Selecting and Evaluating a Referral Laboratory; Approved Guideline (1998)**. This guideline provides an outline of reasons and criteria for choosing a referral laboratory. A checklist for evaluating potential referral laboratories is included to assist in the decision process.

**MM1-A** **Molecular Diagnostic Methods for Genetic Diseases; Approved Guideline (2000).** This document provides guidance for the use of molecular biologic techniques for clinical detection of heritable mutations associated with genetic disease.

**MM2-A** **Immunoglobulin and T-Cell Receptor Gene Rearrangement Assays; Approved Guideline (1995).** This document provides guidelines for conducting molecular tests of immunoglobulin and T-cell receptor gene arrangements.

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

**MM4-A** **Quality Assurance for Immunocytochemistry; Approved Guideline (1999).** This document provides recommendations for the performance of immunocytochemical assays on cytologic and surgical pathology specimens. It is intended to promote a better understanding of the requirements, capabilities, and limitations of these diagnostic methods, to improve their intra- and interlaboratory reproducibility, and to improve their positive and negative predictive values in the diagnosis of disease.

**MM7-P** **Fluorescence *in Situ* Hybridization (FISH) Methods for Medical Genetics; Proposed Guideline (2001).** This document addresses FISH methods for medical genetic determinations, identification of chromosomal abnormalities, and gene amplification. Recommendations for probe and assay development, manufacture, qualification, verification, and validation; instrument requirements; quality assurance; and evaluation of results are also included.

* 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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**NOTES**

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San Francisco General Hospital (CA)
Santa Clara Valley Medical Center (CA)
Sentara Williamsburg Community Hospital (VA)
Seoul Nat'l University Hospital (Korea)
South Bend Medical Foundation (IN)
So. California Permanente Medical Group
Southwest Texas Methodist Hospital (TX)
South Western Area Pathology Service (Australia)
Southern Maine Medical Center
Spartanburg Regional Medical Center (SC)
Specialty Laboratories, Inc. (CA)
Stanford Hospital and Clinics (CA)
State of Washington Department of Health
Stony Brook University Hospital (NY)
Stormont-Vail Regional Medical Center (KS)
Sun Health-Boswell Hospital (AZ)
Sunnybrook Health Science Center (ON, Canada)
Swedish Medical Center – Providence Campus (WA)
Temple University Hospital (PA)
Tenet Odessa Regional Hospital (TX)
The Toledo Hospital (OH)
Touro Infirmary (LA)
Trident Regional Medical Center (SC)
Tripler Army Medical Center (HI)
Truman Medical Center (MO)
Tuenmun Hospital (Hong Kong)
UCLA Medical Center (CA)
UCSF Medical Center (CA)
UNC Hospitals (NC)
University College Hospital (Galway, Ireland)
University Hospital (Gent) (Belgium)
University Hospitals of Cleveland (OH)
University of Alabama-Birmingham Hospital
University of Alberta Hospitals (Canada)
University of Colorado Health Science Center
University of Chicago Hospitals (IL)
University of Illinois Medical Center
University of the Ryukyus (Japan)
University of Virginia Medical Center
UZ-KUL Medical Center (Belgium)
VA (Hines) Medical Center
VA (Kansas City) Medical Center (MO)
VA (San Diego) Medical Center (CA)
VA (Tuskegee) Medical Center (AL)
Vejle Hospital (Denmark)
Virginia Department of Health
ViroMed Laboratories (MN)
Warren Hospital (NJ)
Washington Adventist Hospital (MD)
Washoe Medical Center Laboratory (NV)
Waterford Regional Hospital (Ireland)
Wellstar Health Systems (GA)
West Jefferson Medical Center (LA)
West Shore Medical Center (MI)
Wilford Hall Medical Center (TX)
William Beaumont Army Medical Center (TX)
William Beaumont Hospital (MI)
William Osler Health Centre (Brampton, ON, Canada)
Winn Army Community Hospital (GA)
Winnipeg Regional Health Authority (Winnipeg, Canada)
Wishard Memorial Hospital (IN)
Yonsei University College of Medicine (Korea)
York Hospital (PA)


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