

Reference Leukocyte Differential Count (Proportional) and Evaluation of Instrumental Methods

A reference method for the evaluation of automated differential counters, based on the visual differential count.



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Reference Leukocyte Differential Count (Proportional) and Evaluation of Instrumental Methods

ABSTRACT

NCCLS document H20-A, *Reference Leukocyte Differential Count (Proportional) and Evaluation of Instrumental Methods*; Approved Standard, evaluates automated and semiautomated hematology instruments for their capability to perform an acceptable leukocyte differential count. The standard focuses on leukocytes found in the peripheral blood films. The standard presents a detailed description of an acceptable manual-visual leukocyte differential count which serves as the reference for the instrumental differential counter. The types of abnormalities to be included are outlined.

A statistical method is outlined, also, allowing for the determination of the performance of the test method in qualitative as well as quantitative abnormalities.

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Reference Leukocyte Differential Count (Proportional) and Evaluation of Instrumental Methods

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TABLE OF CONTENTS

ABSTRACT	i
COMMITTEE MEMBERSHIP	vii
FOREWORD	ix
1.0 INTRODUCTION	1
1.1 Automated White Cell Differential Counters	1
1.2 Classification of Automated Devices for Differential Leukocyte Counts	1
1.3 Limitations	1
1.4 Causes of Variability in Leukocyte Counts	2
2.0 SCOPE	2
3.0 PRINCIPLES	2
3.1 Selection of Reference Method	2
3.2 Safety and Effectiveness of Differential Counters	2
3.3 Performance Testing	3
4.0 DEFINITIONS	3
5.0 REFERENCE LEUKOCYTE DIFFERENTIAL COUNT	6
5.1 Specimen Collection	6
5.2 Blood Film Preparation	7
5.3 Requirements for an Acceptable Blood Film	7
5.4 Romanowsky Staining	8
5.5 Peripheral Blood Nucleated Cells to be Included in the Differential Count	8
5.6 Protocol for Examining Blood Film	12
6.0 EXPERIMENTAL DESIGN	19
7.0 SAMPLE PREPARATION	22
7.1 Preparation	22
7.2 Labeling	22
8.0 DATA COLLECTION	22
8.1 Performance of Tests	22
8.2 Data Acquisition	23
8.3 Imprecision Data Acquisition	24
9.0 CLINICAL SENSITIVITY STUDY	27
9.1 Reference (Normal) Values	28
9.2 Sensitivity for Finding Abnormal Samples	30
10.0 DATA ANALYSIS	31

10.1	Comparison of Methods (Inaccuracy)	31
10.2	Imprecision	34
10.3	Clinical Sensitivity	35
 REFERENCES		41
 SUMMARY OF COMMENTS		43
 RELATED NCCLS PUBLICATIONS		49

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FOREWORD

This document represents the efforts of the NCCLS Subcommittee on Qualitative Cellular Hematology which was appointed to develop a standard for leukocyte differential counting.¹ Particularly vigorous discussion centered on several topics, including the choice of a reference method and statistical methods to evaluate instrument or routine method performance. We do not intend the method to be a field method, although many of its features should be incorporated into the field methods to improve their performance.

Differential leukocyte counts (either visual² or instrumental³) should have medically acceptable false negative rates for unusual or abnormal conditions. In addition, however, they would be expected to have economically feasible false positive rates.

This standard presents a detailed description of an acceptable manual-visual leukocyte differential count which will serve as the reference for the instrumental differential counter. A total of 100 normal specimens serves as the basis for the generation of reference values for the leukocytes normally found in the peripheral blood. One hundred abnormal specimens are also compared to the reference population in the clinical sensitivity study. The types of abnormalities to be included are outlined in the standard. Finally, a statistical method is outlined which allows for the determination of the performance of the test method in qualitative as well as quantitative abnormalities.

The method as outlined is laborious and time-consuming. In its complete form it may not be acceptable to many laboratories. There are simplified versions of this standard which have been published. That method requires relatively few specimens and no complicated statistical procedures.⁴

The subcommittee felt that the wedge technique should be used to prepare peripheral blood films. Special studies done for the subcommittee confirmed the acceptability of the wedge film.

Statistical studies are somewhat confounded by the commonly used method of reporting differentials (i.e., the proportional or percentage system). In the future, absolute concentrations of circulating leukocytes will likely become the preferable method of reporting.

Another area for considerable discussion, within the committee and in the entire field of laboratory medicine, is defining the "differential blood count." Definitions vary from an enumeration of the major leukocyte groups (granulocytes, lymphocytes, and monocytes) to a very comprehensive review of all of the so-called formed elements, including erythrocytes and platelets. This document is limited to leukocytes normally found in the peripheral blood, including subdifferentiation of lymphocytes and neutrophils, plus the requirement that an "other" category be included for all other nucleated cells found in the peripheral blood. We presume that subsequent committee efforts will extend and refine this work.

Much of the information included in this document can be useful to the routine hematology laboratory either in the production of accurate white cell differential counts or for incorporation into quality control procedures. As an example, the production of good peripheral blood films and their evaluation have been detailed in this document.

The tentative edition of H20 was widely reviewed in the laboratory community and generated a variety of remarks. In addition, an international conference centered around this standard was hosted by NCCLS in November 1984 in Washington, D.C.⁵ The subcommittee thanks everyone for their recommendations. Each comment was carefully reviewed and changes were made in the document where appropriate. The statistical analyses have been simplified considerably. Not all viewpoints could be accommodated, however; all comments and subcommittee responses are included at the end of the document.

KEY WORDS

Basophil, differential counting, eosinophil, leukocyte, lymphocyte, lymphocyte (variant form), monocyte, neutrophil (band form), neutrophil (segmented), peripheral blood film (differential leukocyte count).

UNIVERSAL PRECAUTIONS

Universal precautions should be observed when collecting blood specimens. Specimens from any patient could be infected with human immunodeficiency virus (HIV) or hepatitis B virus (HBV). Proper blood collection techniques should be followed to minimize risk to the laboratory staff, and gloves should be worn when appropriate. (see NCCLS Document [M29-T2](#), *Protection of Laboratory Workers from Infectious Disease Transmitted by Blood, Body Fluids, and Tissue—Second Edition*; Tentative Guideline). Spinner instruments used to prepare monolayered blood films are not recommended due to the dangers of aerosolization of the blood sample during slide preparation.

REFERENCE LEUKOCYTE DIFFERENTIAL COUNT (PROPORTIONAL) AND EVALUATION OF INSTRUMENTAL METHODS

1.0 INTRODUCTION

1.1 Automated White Cell Differential Counters

Automated white cell differential counters relieve the clinical laboratory of a labor-intensive activity.⁶ Because predetermined criteria are substituted for visual perception of laboratory personnel with varying skill and training, automation should improve precision of the results. An opportunity also exists to improve the precision of the results by performing counts on many more cells than can be conveniently classified by human visual examination.

1.2 Classification of Automated Devices for Differential Leukocyte Counts

1.2.1 *Differential Counting Techniques*

There are several different techniques for differential counting, including computerized image processing, flow methods, and other methods. The leukocyte types identified by these techniques are comparable, although not always identical.

1.2.2 *Automated Devices*

Several different levels of automated devices have been developed. Examples and intended uses of these different automated devices include:

- (1) Automated cell locators and classifiers that tabulate the usually circulating cells and flag for review any abnormal leukocytes or other variations from normal
- (2) Classifiers of normal and abnormal leukocytes, which are suitable for screening purposes
- (3) Classifiers of normal and abnormal leukocytes, which are suitable for diagnostic purposes (i.e. flagging systems)
- (4) Devices for qualitatively and/or quantitatively determining patterns (size, shape, and/or staining) of formed elements in human blood (leukocytes, erythrocytes, and/or platelets) in addition to one of the preceding uses.

1.3 Limitations

This publication is limited to cells that normally circulate in the human bloodstream plus a category for other cells that does not necessarily require identifying these cells. Thus, flagging clinically abnormal samples for visual review is an integral part of performance evaluation. Opinions differ regarding acceptable reference ranges for leukocytes, complicating this identification. Disagreement also exists on the desirability of proportional (percentage) as compared to absolute counts. To address these considerations fairly, methods in this standard must be used with reference range criteria determined by the user.

1.4 Causes of Variability in Leukocyte Counts

1.4.1 Sources of Variation

Sources of variation for a laboratory measurement may be divided into preanalytical, analytical, biological-physiological, and pathological. One or a combination of these variables may interfere.

1.4.2 Routine vs. Optimal Testing Times

Information gathered during routine testing may differ from data compiled under optimal conditions and supervision. Both sets of data are valid samplings of truth; however, performance data gathered during optimal testing times is not necessarily reproduced during routine testing. These methods of evaluation more closely resemble optimal than routine conditions.

2.0 SCOPE

- 2.1 These recommendations cover performance testing of leukocyte differential counting. Only those common leukocyte types normally found in individuals will be addressed. These cell types are: neutrophils (segmented), neutrophils (band forms), lymphocytes (normal), lymphocytes (variant forms), monocytes, eosinophils, and basophils. If not identified, the system should appropriately flag other cells as: abnormal, suspect or unclassified, or as nucleated red blood cells.

Some devices may group several cell types into a single category; for example, segmented and band neutrophils, eosinophils, and basophils may be combined as granulocytes.

3.0 PRINCIPLES

3.1 Selection of Reference Method

3.1.1 Accuracy

Automated methods to be tested using these principles are independent of external methods of evaluation in all respects except accuracy. To measure accuracy, a reference method is given which in itself must be controlled. The reference method is defined in [Section 5.0](#).

3.1.2 Subjectivity

It is recognized that the reference method depends on human expertise and can be influenced by the subjective nature of the test. However, by formulating the reference method to involve a group of human observers, the subjective nature of the testing can be at least partially overcome.

The subjective nature of the reference method should be remembered when making comparisons with the automated technique. For purposes of this standard, the reference method, with its known imprecision and biases, represents the best estimate of truth for each sample.

3.2 Safety and Effectiveness of Differential Counters

The goal of each test of performance must be the safety and effectiveness of the method with respect to its intended use. A manufacturer, in claims for automated

analyzers, will state one or more intended uses of the instrument. The claim(s) are then tested.

3.3 Performance Testing⁷

Tests of performance must include:

3.3.1 *Comparison*

Comparison with the reference method.

3.3.2 *Internal Consistency*

Tests of internal consistency, including the imprecision of the entire method and stability of the various parameters over time.

3.3.3 *Establishing Reference (Normal) Ranges*

A means of establishing reference (normal) ranges. These may or may not be equivalent to the reference (normal) ranges obtained on the identical specimens at the same time by the reference method.

3.3.4 *Clinical Sensitivity*

Using established reference (normal) ranges ([see Section 3.3.3](#)) to test the sensitivity to abnormal samples of the automated instrument at the level claimed by the manufacturer (i.e., clinical sensitivity of intended use).

3.3.5 *Safety and Effectiveness*

A means of confirming that the limitations of the automated instrument are consistent with safety and effectiveness in the intended use.

3.3.6 *Cell-by-Cell Performance Evaluation*

Evaluation of cell-by-cell performance of a device only if the manufacturer claims such performance.

3.3.7 *Tabulated Data vs. Manufacturer's Claims*

The tabulated data must be compatible with the manufacturer's claims. For example, a manufacturer claiming a required review for specific cells (abnormals or suspects) will have flagging data tabulated. This is to be decided on an individual instrument basis.

4.0 DEFINITIONS

- (1) **Abnormalities.** An abnormality may be a clinically significant alteration in the distribution of mature cell types, or the presence of immature or abnormal cell types in a clinically significant concentration.
- (2) **Arbitrator.** A qualified examiner ([see Qualified Examiner](#) below), frequently with additional expertise and experience, who will resolve disagreements between test and reference methods and between the results of two qualified examiners.

- (3) **Automated System (Device).** An inclusive term to denote the instrument, reagents, and methods of the device under study.
- (4) **Clinical Sensitivity.** A test's ability to identify individuals with the illness being tested for. In the context of this document, it is the test method's ability to obtain positive results in concordance with positive results obtained by the reference method.⁸
- NOTE:** If the true sensitivity of a device is better than the reference method, its apparent specificity will be less and the level of apparent false positives will be greater.
- (5) **Clinical Specificity.** A test's ability to recognize individuals without the illness being tested for. In this document, it is the test method's ability to obtain negative results in concordance with negative results obtained by the reference method.⁸
- (6) **Evaluation of Formed Elements.** An inclusive term encompassing:
- (a) Tabulating leukocytes in a representative sample of human blood by a standard classification scheme, sometimes called a differential or "diff"
 - (b) A statement on the presence or absence of certain erythrocyte abnormalities
 - (c) A statement on the sufficiency, morphology, excess, or lack of platelets in the sample.
- (7) **False Positive; False Negative.** A false positive (FP) is a positive test in a subject in whom the disease is absent. In the context of this document, a false positive is a positive result by the test method where the reference method result is negative on the same sample. A false negative (FN) is a negative test in a subject in whom the disease is present. In this document, it is a negative result by the test method on a sample where the reference method result is positive.⁸
- (8) **False Positive Ratio (or fraction); False Negative Ratio (or fraction).** (See NCCLS document [GP10-P](#).) The conventional method for determining these rates is presented in the following table and equations.⁸

Test Method Results

Reference Method Results	Test Method Results	
	Positive	Negative
Positive	TP (True Positive)	FN (False Negative)
Negative	FP (False Positive)	TN (True Negative)

$$\text{False Positive ratio} = \frac{\text{FP}}{\text{TN} + \text{FP}} \times 100 = \quad \%$$

$$\text{False Negative ratio} = \frac{\text{FN}}{\text{FN} + \text{TP}} \times 100 = \quad \%$$

The ratio of false positives and false negatives in flagging abnormalities under routine use in the intended setting for the devices is made for either:

- (a) Overall laboratory performance, or
 - (b) A specified abnormality or set of abnormalities.
- (9) **Flagging.** Identifying a sample or blood film for further attention or review.
 - (10) **Imprecision.** Standard deviation or coefficient of variation of the results in a set of replicate or duplicate measurements. The mean value and number of replicates must be stated; the experimental design must be described so that other workers can repeat it. This is particularly important when a specific term is used to denote a particular type of imprecision, such as short term, or long term.
 - (11) **Inaccuracy.** Numerical difference between the mean of a set of replicate measurements and the true value as determined by a group of experts. This difference (positive or negative) may be expressed in the units in which the quantity is measured, or as a percentage of the true value. In this document, inaccuracy is also expressed as the mean difference together with the 95% confidence limits.
 - (12) **Interference.** The effect of a component, which does not by itself produce a reading, on the accuracy of measurement of another component.
 - (13) **Leukocyte Differential Count.** Determining the proportion or absolute concentration of defined classes or subsets of leukocytes in a sample of peripheral blood. In this standard all counts are given in proportional terms.
 - (14) **Operator(s).** The person or persons in control of the sample or prepared sub-sample during performance of the test. Operators are required for the reference method, routine method, and automated method.
 - (15) **Parameter.** An output of the device being tested. This may be a single number, a numeric frequency distribution, or a flag for a specific cell or class of cells. A general sample review requirement, or a combination of outputs which, according to the manufacturer's instructions, is a flag for sample review.
 - (16) **Prepared Sub-Sample.** A portion of the test sample which has been treated according to instructions of the reference method or the manufacturer, and made ready for further processing in the system. Numerous sub-samples may be prepared by way of a variety of methods from each sample.

- (17) **Qualified Examiner.** A person with special training and recognized skills in peripheral blood cell morphology, and who has been qualified as detailed in [Section 5.6.3 through 5.6.5](#).
- (18) **Reference Method.** (The following definition pertains to the context of this standard only.) A carefully controlled set of specified procedures including sample preparation, staining, classification, and tabulation of each sample. It is a statistically devised protocol aimed at optimizing the chance of a proper determination of truth. All leukocytes are classified sequentially by a qualified examiner with special skills in morphologic hematology; therefore, interaction of the parameters is a component of the reference method. Although the reference method is considered to be accurate, it may still be imprecise. This imprecision can be reduced by carrying out the procedure in duplicate and averaging the results.
- (19) **Routine Examiner.** A person of average training and skill, performing the routine laboratory method.
- (20) **Routine Laboratory Method.** That method being used routinely in the laboratory, in contrast to the reference method (see [Reference Method](#) above) which is not ordinarily used in routine laboratory operations.
- (21) **Sample.** The appropriately representative part of a specimen ([Section 5.1.1](#)) used in the analysis. This sample may be named a test sample when it is necessary to avoid confusion with the statistical term "random sample from a population."
- (22) **Sensitivity.** The ability of an analytical method to detect small quantities of the measured component. The unit is the unit of measurement.
- (23) **Specimen.** A quantity of blood taken to perform laboratory tests to show or determine the character of the whole blood volume.
- (24) **Specificity.** The ability of an analytical method to determine, within stated concentration limits for which the method is valid, solely the component(s) it purports to measure. Specificity has no numerical value. It is assessed by the evidence available on the extent to which the components contribute to the result.
- (25) **Test Method.** The method to be compared with the reference method (i.e., either the routine laboratory method or automated method).

5.0 REFERENCE LEUKOCYTE DIFFERENTIAL COUNT

To evaluate any leukocyte differential count method, or automated leukocyte differential counter, it is necessary to define a comparative method or reference leukocyte differential count. This reference method deals only with leukocytes.

5.1 Specimen Collection

Whole venous blood collected in tripotassium ethylenediamine tetra-acetate (K_3EDTA) 1.5 ± 0.15 mg (in liquid or powder form) per mL is the required specimen,⁹ (see NCCLS document [H3-A3, Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture—Third Edition](#); Approved Standard). Alternatively, blood collected by skin puncture may be used, but only if it can also be used on the auto-mated differential counter or the leukocyte differential count method being evaluated (see NCCLS document [H4-A3, Procedures for the Collection of Diagnostic Blood Specimens by Skin Puncture—Third Edition](#); Approved Standard). Universal Pre-cautions should be followed. (See NCCLS document [M29-T2](#) for further information.)

5.1.1 *Specimen Condition*

Macroscopically visible clots are cause for rejection of the specimen for analysis. Microscopically visible platelet clumps consisting only of platelets are acceptable, provided their presence is noted; if the automated system is claimed to flag platelet clumps, this provides a test sample for this capability.

Record the total leukocyte count. Record any abnormal condition of the specimen, such as lipemia or hemolysis in the supernatant plasma, at the end of the testing day. It is inadvisable to let the cells settle or to centrifuge the specimen before analysis to observe the supernatant plasma.

5.2 **Blood Film Preparation**

- (1) Prepare three blood films from each specimen on clean, dry and dustfree 25 x 75 mm (1 x 3 in), 0.8 to 1.2 mm thick, glass microscope slides of good quality. Label the slides A, B, and "spare." Two blood films will be used for the procedure and the third will be kept as a spare. [If the blood is leukopenic, prepare a larger number of blood films (e.g., six).]

- (2) Prepare blood films within 4 h of the blood collection in K₃ EDTA.

NOTE: Do not store blood in the refrigerator. Adequate mixing (20 complete inversions by hand) is necessary, before blood film preparation.

- (3) Prepare blood films using the wedge-pull film technique. Place one drop (approximately 0.05 mL) of well-mixed blood near one end of a glass microscope slide. Hold a second, narrower spreader slide with polished edges at about a 45° angle and immediately draw into the drop of blood. Allow the blood to spread almost to the width of the slide. Then rapidly and smoothly push the spreader slide to the opposite end of the slide, pulling the blood behind it.
- (4) Stain the film within one hour of preparation with a Romanowsky stain, containing fixatives; or fix within one hour with "water-free" (i.e., <3% water) methanol for later staining.

5.3 **Requirements for an Acceptable Blood Film¹⁰**

5.3.1 *Desirable Qualities of a Blood Film*

- (1) Sufficient working area
- (2) Minimum 2.5 cm in length terminating at least 1 cm from the end of the slide
- (3) Gradual transition in thickness from the thick to thin areas, ending in a squared or straight edge ([Figure 1](#))
- (4) Acceptable morphology within working area
- (5) Narrower than the slide on which the film is spread, with smooth continuous side margins that are accessible for oil immersion examination
- (6) No artifact introduced by the technique

- (7) Minimum distributional distortion
- (8) A far end that becomes gradually thinner, without grainy streaks, troughs, or ridges, all of which indicate an increased number of leukocytes carried into this area.

It is recognized that less than optimal films occur in cases of anemia or polycythemia or in cases with abnormal plasma proteins (e.g., in myeloma, cold agglutinin disease).

5.4 Romanowsky Staining

A Romanowsky stain is any stain containing methylene blue and/or its products of oxidation (azure B), and a halogenated fluorescein dye, usually eosin B or Y.

The reference differential count preparations require staining with Romanowsky stain. Optimal staining of the cellular elements greatly helps in accurately identifying both mature and immature leukocytes, as well as abnormal cells.

5.4.1 *The Romanowsky Effect*

Successful stains consistently produce the Romanowsky effect, the typical coloration of certain cell components being produced by the combined action of the dyes noted previously at the appropriate pH (6.4 to 7.0). These cell components include leukocyte nuclei and neutrophil-specific granules. Successful stains impart characteristic colors, typically blues and pinks, to other cell components.

5.4.2 *Acceptable Leukocyte Condition*

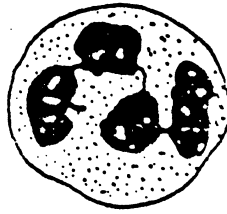
The leukocytes should be well preserved, and anticoagulant effects such as excessive vacuolization or changes in nuclear shape must be minimal. Less than 2% of the leukocytes may be smudged, except in some lymphoproliferative disorders.

5.4.3 *Reproducible Staining*

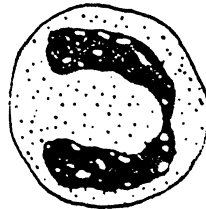
There must be reproducible staining of granulocytic, monocytic, lymphocytic and other cells with clear nuclear-cytoplasmic demarcation, distinct nuclear chromatin patterns, and cytoplasmic color differences.

5.5 Peripheral Blood Nucleated Cells To Be Included in the Differential Count

A variety of nucleated cells occurs in peripheral blood; the following types are commonly observed on a blood film properly prepared as described in [Section 5.2](#).¹¹ A schematic representation of each white blood cell normally found in the peripheral blood is included with each description.

5.5.1 Neutrophil, Segmented Form (Polymorphonuclear Leukocyte)

- (1) Round, and approximately 15 μm in diameter.
- (2) Cytoplasm stains light pink with specific or secondary granules of fairly uniform size.
- (3) Specific granules are evenly distributed, variable in number, and pink to lavender in color.
- (4) The nucleus is lobulated; the lobes are connected by thread-like filaments.
- (5) The elongated nucleus may be folded over; the lobes may be touching each other or may be superimposed, and various nuclear appendages may be seen.
- (6) Nuclear chromatin forms dark, densely stained blocks separated by a network of lighter purple bands.
- (7) Occasionally, large, dark, coarse cytoplasmic granules are seen in patients with infections or other serious illnesses; these are called toxic granules.

5.5.2 Neutrophil, Band or Stab Form

- (1) Similar to segmented forms in size and in cytoplasmic characteristics; different from segmented neutrophils in that the connection between the end of the beginning lobe formation of the nucleus is band-like rather than a filament. The connecting band, or isthmus, is wide enough to reveal two distinct margins surrounding nuclear material.
- (2) Characteristically, the nucleus is elongated with rounded ends and with an area of pyknosis at each pole.
- (3) The nucleus is curved or sausage-shaped, and the sides are parallel over an appreciable distance.
- (4) Less typical band forms have multiple lobes interconnected by wider bands instead of filaments.

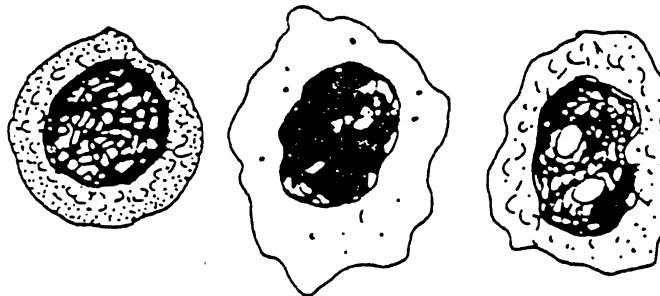
If the examiner is not sure whether a neutrophil is a band form or a segmented form, it is arbitrarily classified as a segmented neutrophil.

5.5.3 *Lymphocytes, Normal Forms*



- (1) Seven to 15 μm in diameter, but generally, 7 to 12 μm .
- (2) Round, or at times slightly indented.
- (3) The basophilic cytoplasm ranges from abundant to sparse and stains pale or bright blue; sometimes the stained cytoplasm appears uneven or bubbly.
- (4) The cytoplasm may contain relatively large azurophilic granules.
- (5) The nuclei vary in size, shape, and chromatin pattern.
- (6) Although usually round, the nuclei may be kidney shaped. Notching or clefts may be seen, or they may be lobulated or folded.
- (7) The chromatin is arranged in densely staining compact blocks separated by lighter tones without sharp demarcation. Sometimes a fine chromatin pattern is seen, with evident nucleoli; at other times the chromatin appears filmy, coarsely granular, or ropy.

5.5.4 *Lymphocytes, Variant Forms*

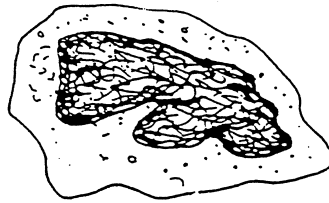


- (1) These cells can be normal physiologic variants or abnormal forms. These cells are large and quite variable in appearance. Several distinct types have been described. The terms "atypical, reactive, Downey cell, virocyte" etc., have been used to identify these cells. Because of confusion about the relationship of these cells to either benign or malignant processes, the subcommittee chose the new term—lymphocytes, variant forms.
- (2) The cytoplasm may be abundant, often appear foamy, or even frankly vacuolated.

- (3) Increased cytoplasmic basophilia may be noted, especially at points of contact with adjacent cells; usually, the cytoplasmic staining ranges from blue-gray to light blue.
- (4) The nuclear chromatin may be dense, lumpy, or "blocked" with clearer areas of parachromatin; nucleoli may be visible.

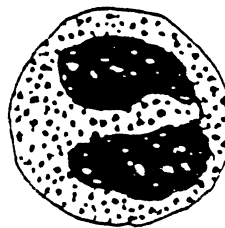
A normal differential count usually includes up to 6% of variant forms. Transitional forms between normal and variant lymphocytes are also found. In children in apparently good health, more immature-appearing lymphocytes with clear nucleoli are sometimes found.

5.5.5 *Monocytes*



- (1) Usually larger than neutrophils, ranging from 12 to 22 μm in diameter.
- (2) Although the majority are round with smooth margins, some monocytes may have one or more large, or multiple smaller, blunt cytoplasmic protrusions.
- (3) The cytoplasm stains gray-blue and contains numerous small, poorly defined granules causing a "ground glass"-like appearance. Sometimes numerous, dust-like and/or discrete azurophilic granules are seen. Vacuoles are common and phagocytosed particles are sometimes seen.
- (4) The nuclei are quite variable in shape and may be round, oval, indented, deeply lobulated, or even segmented. In most cases the nucleus shows some degree of folding or brain-like convolutions.
- (5) The chromatin stains light purple and lacy, although it may be coarse at times.

5.5.6 *Eosinophils*

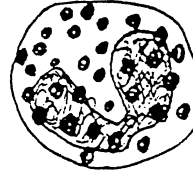


- (1) Eosinophilic granulocytes are slightly larger than neutrophils, usually 12 to 16 μm in diameter.
- (2) The cytoplasm contains many large spherical refractile granules, uniform in size, which stain from bluish-red to bright orange-red.

The granules are usually evenly distributed, fill the cytoplasm but rarely overlay the nucleus.

- (3) The nucleus is usually segmented into two or three lobes, and occasionally more.

5.5.7 *Basophils*



- (1) Basophilic granulocytes are smaller than eosinophils or neutrophils, usually 10 to 14 μm in diameter.
- (2) They are characterized by densely stained, dark violet to purplish black cytoplasmic granules which are variably sized and unevenly distributed. Some granules usually overlay or even partially obscure the nucleus. The granules are water-soluble and therefore only vestiges of granules, sometimes apparently contained within small vacuoles, may be found.
- (3) The nuclei are deeply indented or segmented.

5.5.8 *Other Nucleated Cells Less Commonly Found in Peripheral Blood*

These cell types are described in special hematology texts and atlases, and include "smudges," "basket cells," and other unidentifiable forms. Adding one drop of 22% human albumin to five drops of blood markedly reduces smudge formation. Make the blood film from the albumin-blood mixture.¹²

5.6 Protocol for Examining Blood Film

5.6.1 *Microscopic Examination of the Blood Film*

- (1) The blood film should always be scanned under low power (10x to 40x objective) for unusual or abnormal cells and an acceptable cell distribution. Most 100x oil immersion objectives are capable of resolving cytoplasmic granules and neutrophilic filaments.
- (2) Extend the examination from the area where approximately 50% of the erythrocytes overlap to the region where erythrocytes show a strong tendency to linear orientation, which, in even thinner parts of the film, causes separate streaks known as the "feather edge."

In the examination area of a normal patient specimen, the average size of erythrocytes (measured by averaging the longest and shortest diameter, using an ocular micrometer) should be constant to within 0.3 μm , and the standard deviation (SD) of 200, more or less adjacent, single erythrocytes should not exceed 0.6 μm .

- (3) A minimum of 300 leukocytes should be within the acceptable working area, when the total leukocyte count is no less than 4×10^9 per L.
- (4) The neutrophils, monocytes, and lymphocytes should appear evenly distributed in the "usable" fields of the film (see Section 5.6.1(2) above).

When the total leukocyte count is within the normal range, the number of white cells per 100X field at the tail area should not exceed 2 to 3 times the number per field in the body of the film.

The side edges should contain less than 2 to 3 times the number of cells per 100X field than the body of the film.

- (5) Except for certain forms associated with pathological states (e.g., chronic lymphocytic leukemia) less than 2% of the white cells should be disrupted or nonidentifiable forms. Only if the disrupted cell is still clearly identifiable (e.g., an eosinophil) should it be included in the differential count. Classify nonidentifiable disrupted cells (smudges or baskets) as "other" and include a comment on the laboratory report (see Section 5.5.8).

5.6.2 ***Counting Procedure***

- (1) Use the "battlement" track for this examination (see Figure 1). Each identified cell must be placed into one of the following categories: neutrophil, segmented; neutrophil, band; lymphocyte, normal; lymphocyte, variant; monocyte; eosinophil; basophil; other nucleated cells (except nucleated red cells); see Section 5.6.2 (3) and (4) below. Include distorted cells that are clearly identifiable in the appropriate classification.

FIGURE 1

Push Wedge Film

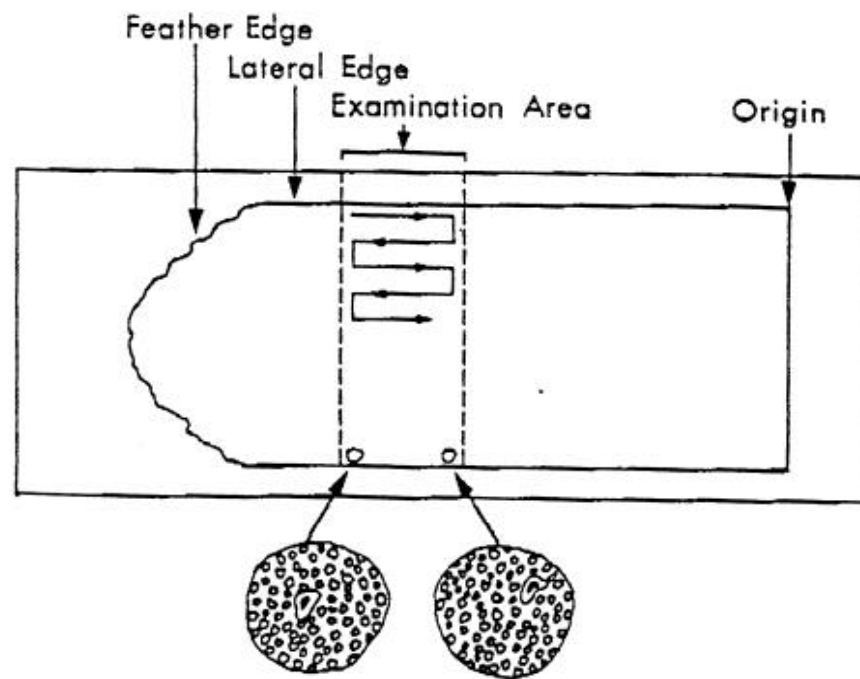


Illustration of a properly made push wedge film of peripheral blood, showing the battlement pattern for film examination.

Reprinted with permission from *Lab. Medicine* 11:371-375, 1980.

- (2) On each slide, 200 leukocytes should be counted. If the blood is leukopenic, process additional slides in parallel.
- (3) Express the results of the differential count as a fraction (i.e., percentage of all the leukocytes counted).
- (4) Count any nucleated erythroid cells present and express the result as the number per 100 leukocytes counted.

5.6.3 ***Qualifying the Blood Film Examiner***

5.6.3.1

Examiner's Experience

Training and experience in examining immature and abnormal cell morphology are essential. The examiner must be able to classify all common leukocytes and should know most leukocyte variations, both congenital and acquired.

5.6.3.2 *Examiner's Attitude*

The attitude, motivation, and concentration of the examiner are key factors in performance. Because of the rote nature of the differential counting procedure, the examiner's performance can be influenced by the importance placed on the differential count, the presence of distractions in the laboratory environment, and the workload. For reference work, the examiner should perform no more than 15 to 25 200-cell differential counts each day.

5.6.4 ***The Qualification Blood Films***

5.6.4.1 *Qualification of Examiners*

Ten donor specimens are selected. The specimens should include among them cells of all seven types included in [Section 5.5](#) (segmented neutrophils, band neutrophils, normal lymphocytes, variant lymphocytes, monocytes, eosinophils and basophils). There should also be at least one specimen containing a small proportion of nucleated red cells and one specimen containing a small proportion of immature white cells.

Five blood films are prepared from each specimen according to the procedure described in [Section 5.2](#) and stained as per [Section 5.4](#), labeled uniquely in a way which keeps examiners blinded as to the source of the sample. The slides are organized into five sets. Each set contains a slide of each blood specimen.

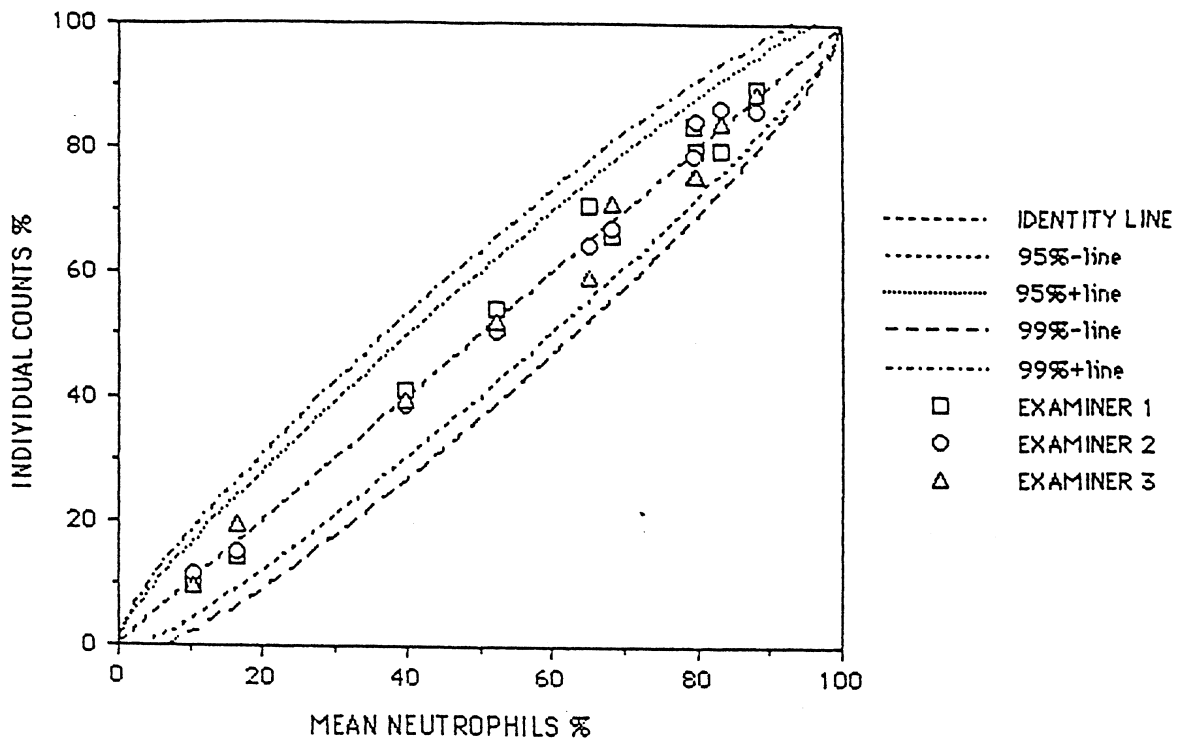
5.6.5 **The Qualification Process**

A set of qualification blood films is distributed to each participating examiner. They are requested to perform a 200-cell differential count and submit the results identified by coding number, to the coordinator of the qualifying procedure.

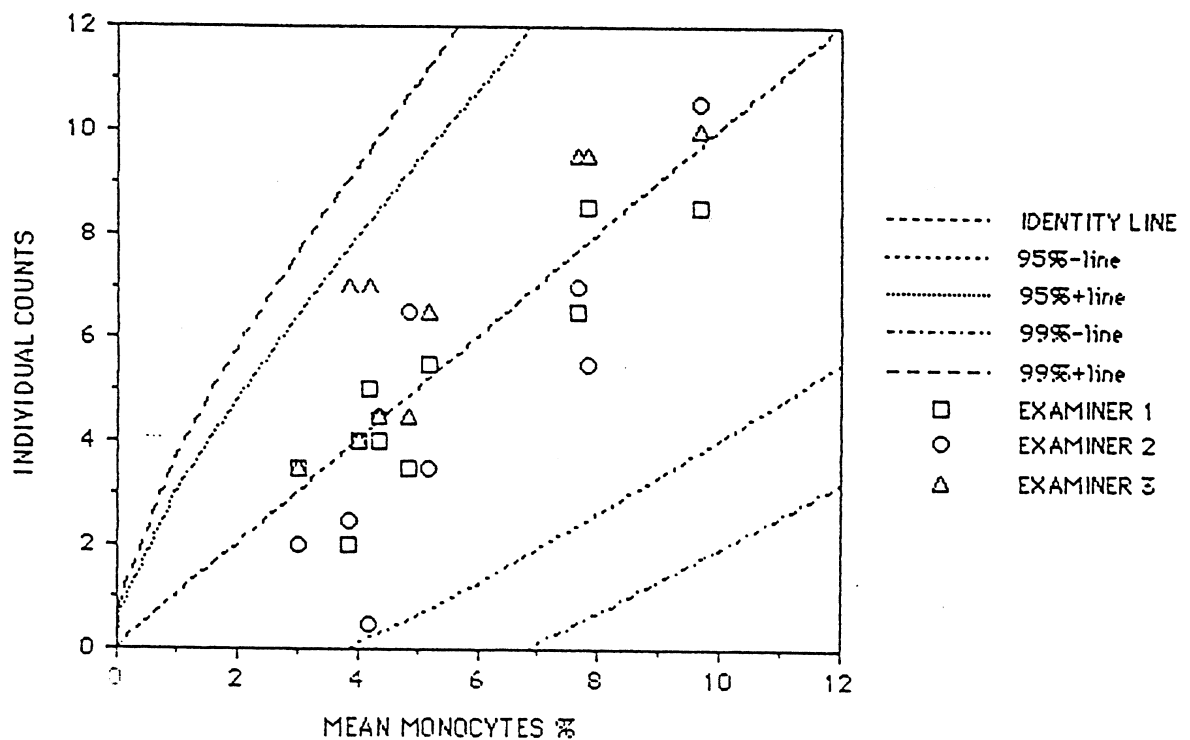
The coordinator decodes the results and calculates the mean relative concentration for each cell type per sample. A graph is prepared for each cell type ([see Figure 2](#)). The abscissa (X axis) represents the mean relative concentration (percentage).

FIGURE 2

GRAPH OF QUALIFYING COUNTS FOR NEUTROPHILS



GRAPH OF QUALIFYING COUNTS FOR MONOCYTES



This group mean represents the laboratory qualified examiners mean, and should be similar to another laboratory's qualified examiner mean. The

ordinate (Y axis) represents the individual technologist results. Superimposed on this graph are two envelopes representing theoretical 95% and 99% confidence bands, derived from the formula for the Standard Error of a Proportion. (See Table 1.)

TABLE 1

Work Table for Generation of Confidence Bands

95% AND 99% CONFIDENCE BANDS DERIVED FROM SE_p

% CELLS	p	q	SE_p	95% SE_p	99% SE_p	LOW 95%	HIGH 95%	LOW 99%	HIGH 99%
0	0	0	0	0	0	0	0	0	0
1	1	99	1	1	2	0	2	0	3
2	2	98	1	2	3	0	4	0	5
4	4	96	1	3	4	1	7	0	8
6	6	94	2	3	5	3	9	1	11
8	8	92	2	4	5	4	12	3	13
10	10	90	2	4	6	6	14	4	16
12	12	88	2	5	6	7	17	6	18
14	14	86	2	5	7	9	19	7	21
16	16	84	3	5	7	11	21	9	23
18	18	82	3	5	7	13	23	11	25
20	20	80	3	6	8	14	26	12	28
25	25	75	3	6	8	19	31	17	33
30	30	70	3	6	9	24	36	21	39
35	35	65	3	7	9	28	42	26	44
40	40	60	3	7	9	33	47	31	49
45	45	55	4	7	9	38	52	36	54
50	50	50	4	7	10	43	57	40	60
55	55	45	4	7	9	48	62	46	64
60	60	40	3	7	9	53	67	51	69
65	65	35	3	7	9	58	72	56	74
70	70	30	3	6	9	64	76	61	79
75	75	25	3	6	8	69	81	67	83
80	80	20	3	6	8	74	86	72	88
85	85	15	3	5	7	80	90	78	92
90	90	10	2	4	6	86	94	84	96
95	95	5	2	3	4	92	98	91	99
100	100	0	0	0	0	100	100	100	100

Also see "Confidence Limit Table" in Geigy Scientific Tables, Vol. 2, p. 102, 8th Edition, 1982. Ciba Geigy, Basile, Switzerland.

Standard error of Proportion:

$$SE_p = \left[\frac{p \times q}{n} \right]^{\frac{1}{2}}$$

95% Confidence Interval for a single proportion:

$$p \pm 1.96 \left[\frac{p \times q}{n} \right]^{\frac{1}{2}}$$

99% Confidence Interval for a single proportion:

$$p \pm 2.57 \left[\frac{p \times q}{n} \right]^{\frac{1}{2}}$$

where:

n = 200 (cells observed per examiner)

p = mean value (of two or more examiners)

q = 100 - p

Let Student factor (S_t) be the 95th (or 99th) percentile of the t distribution with 199 degrees of freedom.

For a 95% confidence limit use $S_t = 1.96$

For a 99% confidence limit use $S_t = 2.57$.

An alternate method based upon the statistical studies of Rümke can be used to delineate the appropriate confidence bands.¹³

5.6.5.1 *Interpretation*

Based on the assumption of Gaussian distribution, 5% of the individual technologist results may fall outside the 95% envelope but realistically none should exceed the 99% limits. (Statistically, 1 case in 100 may exceed the 99% limit.)

General failure of the data set to conform to this empirical rule indicates the presence of a procedural or operational error (e.g., sample identification error, poor slide preparation, reading in an inappropriate area of the slide, or cell classification errors). The qualification exercise should be repeated after the possible sources of error have been carefully reviewed. Failure to achieve conformity to this statistical rule casts serious doubt on the validity of any subsequent reference results.

The coordinator should carefully examine each graph for patterns. The individual technologist values should present a random pattern. If this is not the case, a technologist bias can be introduced into the evaluation. Corrective action (e.g., training of the technologist, replacement, etc.) should be

implemented and the reference qualification repeated.

The slides are retained as a master set for qualification of any subsequent examiners and may be used for training and qualification of laboratory technologists in the laboratory for differential counting.

6.0 EXPERIMENTAL DESIGN

The method is primarily suited for the analysis of leukocyte types which have a normal range of 5% or more. For cells present in lower concentrations (e.g., basophils), the coefficients of variation are expected to be large. Consider those cell types below this level only in the clinical sensitivity evaluation. If special selected cases are available, tests of imprecision may also be made.

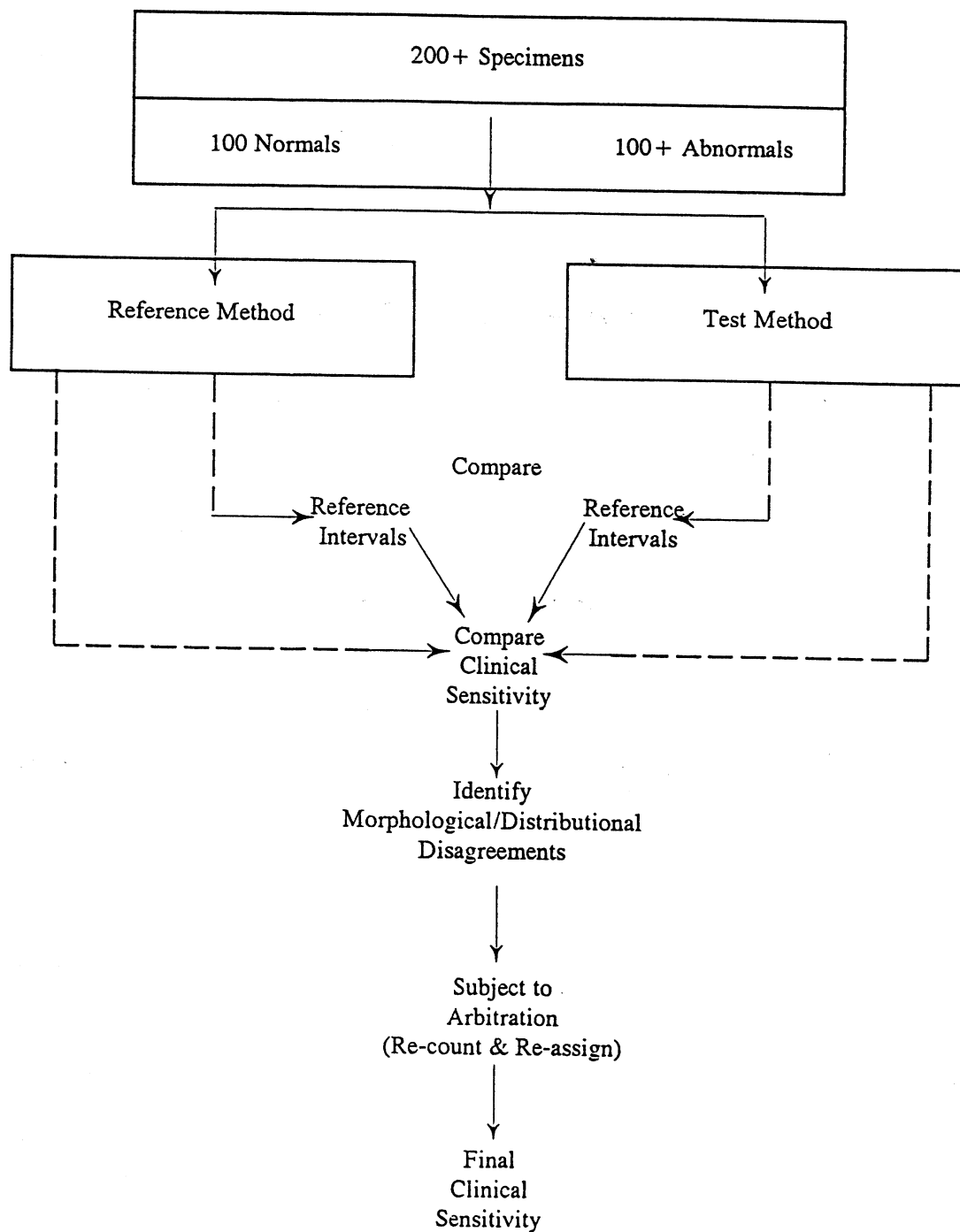
The evaluation protocol has been divided into several major sections including:

- (1) Sample preparation
- (2) Leukocyte comparisons for inaccuracy and imprecision
- (3) Clinical sensitivity, including reference intervals
- (4) Statistical methods.

The sections are interdependent procedurally but each topic will be addressed separately. (See [Figure 3](#) for an overview of the protocol.)

FIGURE 3

Outline of Experimental Protocol for Clinical Sensitivity



If the protocol and specimen collection are carefully planned, this whole study can be carried out on a total of 200 specimens. Of these, 100 specimens must be qualified as normal, and up to 100 must correspond to the needs described in [Section 9](#). (See Table 2.) Realistically, however, an additional number of specimens may be studied in order to include the various clinical conditions (Table 2).

TABLE 2
Specimen Types for Clinical Sensitivity Study

Clinical Condition	Characteristic Leukocyte Differential Count Finding	Absolute Cell Count	Proportional Cell Count*
Acute inflammation	Granulocytosis	$\geq 9.0 \times 10^9/L$	$> 80\%$
Bacterial infection	and/or Left shift** (band-forms)	$\geq 0.9 \times 10^9/L$	$> 6\%$
Chronic inflammation	Monocytosis	$\geq 0.8 \times 10^9/L$	$> 10\%$
Parasitic infection	Eosinophilia	$\geq 0.5 \times 10^9/L$	$> 7\%$
Allergic reaction			
Viral infections	Lymphocytosis	$\geq 3.5 \times 10^9/L$	$> 50\%$
infectious mononucleosis	and/or		
cytomegalovirus infection	Lymphocytes, variant forms**	$\geq 0.7 \times 10^9/L$	
infectious hepatitis			
Aplastic anemia, chemotherapy	Granulocytopenia	$\leq 1.5 \times 10^9/L$	$< 10\%$
HIV infection	Lymphopenia	$\leq 1.0 \times 10^9/L$	$< 7\%$
Acute leukemia	Immature cells, including blasts**	$\geq 0.1 \times 10^9/L$	$> 2\%$
Severe anemia myelophthisic anemia	Nucleated red blood cells**	$\geq 0.02 \times 10^9/L$	$> 2\%$

* In addition to noted absolute counts, the specimens should also have these proportional counts.

** Findings for morphological classification; other findings are considered to be distributional changes. Aim to include at least five cases of each condition in the clinical sensitivity study.

7.0 SAMPLE PREPARATION

7.1 Preparation

- (1) Prepare samples according to the reference method protocol for the reference counts.
- (2) Prepare samples according to the manufacturer's recommendation for the test counts.
- (3) Choose samples for the study at random from the laboratory workload of the testing institution. Include both normal and abnormal samples. The type of specimens required for the clinical sensitivity study are listed in [Table 2](#).

7.2 Labeling

Label the samples uniquely by number in a way which keeps examiners blinded as to source of sample. (See specific labeling instructions in [Section 8.1.6](#).) Keep a log that allows for identification of the patient's chart or history number from the sample label.

8.0 DATA COLLECTION

8.1 Performance of Tests

- (1) The reference method must be performed by qualified examiners ([see Section 5.6.3](#)) who are employed in the testing laboratory.
- (2) The routine differential counts, as performed in the testing institution by technologists (routine examiners), can be studied as one kind of test method.
- (3) Run the test method daily after completing calibration procedures.
- (4) Keep records of control slide runs or control material runs for flow systems as part of the study.
- (5) Take two samples from the blood specimen for each method under study. Methods will include the reference method, the specific test method, and possibly the routine laboratory method if it is also being evaluated. Perform the laboratory method exactly as practiced in the laboratory. If an image analyzer is being tested, prepare blood films according to the manufacturer's instructions using appropriate equipment. Follow the manufacturer's instructions for staining. If a flow analyzer is being tested, run the analyzer according to the manufacturer's instructions.
- (6) Label samples uniquely as they are acquired into the study. Label reference method slides A, B, and spare. For image processing instruments requiring slides, label test method slides (or results) C, D, and spare. (**NOTE:** If it is a flow method, analyze at least 100 normal and abnormal samples in duplicate by the test method, to provide sufficient data for the imprecision study.) If the test method is a flow method, do not run the specimens sequentially but run at random in the daily workload, in accordance with the manufacturer's directions.
- (7) Maintain a log containing data on sample source and complete blood counts.

- (8) Process prepared samples by each method on a daily basis.
- (9) A 400-cell reference differential count is performed on each patient sample. Each of two qualified examiners performs a 200-cell differential on one of two slides according to the reference procedure (see Section 5.0). One examiner uses slide A and another examiner uses slide B. The data must be traceable to each slide and each examiner.
- (10) Perform two individual determinations for the test method on at least 100 samples. If the test method is a flow system, the remaining samples may be analyzed randomly in the daily workload. Process the automated method specimens as required by the manufacturer. Do not process more than 25% of the total study on a single day. The total number of cells included in these determinations should be at least 400, but the exact number will vary from instrument to instrument; flow cytometers usually measure several thousand cells.

Record the exact number of cells used in the determination. (This may require calculation from manufacturer's literature for flow methods.)
- (11) Include samples covering the full range of normal and abnormal counts usually encountered in the clinical decision-making process. Bands are added to total neutrophils for purposes of statistical analysis when the instrument does not report bands as a separate parameter.

8.2 Data Acquisition

- (1) For purposes of microscopic eye count, it is recommended that a slide tray or box is established as a source box for slides to be examined. Randomize the slides so that the duplicates are rarely processed in sequence. Another box is provided for slides that have been examined. Slides are taken at random from the source box by individual examiners who do the differential count; the slides are then returned to the second box. Only data from qualified examiners can be used for the reference method. Each slide (A and B) from each specimen must be examined by one examiner only and the same examiner must not examine the second slide from that specimen.
- (2) Transcribe results from each differential count to the composite data sheets provided for this purpose (see [Table 3](#)). Note that on this data sheet the samples are already listed in order.
- (3) Retain original printouts and result slips in a file. Initials of the technologist generating the results must accompany each record.
- (4) Record total leukocyte counts for all samples. Tabulate records daily on the forms provided which are suitable for computer input ([Table 3](#)). Calculate means of counts A and B for the reference method and of counts C and D if required for the test method. If the test equipment is computer compatible or has data recording capability, the technologist who performs the tests must verify the computer data.
- (5) The tabulated data must be compatible with the manufacturer's claims. For example, a manufacturer claiming a required review for specific cells (abnormals or suspects) will have flagging data tabulated. Decide this on an individual instrument basis.

8.3 Imprecision Data Acquisition

8.3.1 *Short-Term Imprecision of Method Using Routine Samples*

- (1) In this test, samples used are identical to the samples used in [Section 8.1 \(6\)](#) (i.e., each sample is measured twice within the same run).
- (2) Calculate the short term imprecision of either the test or reference procedures (see [Section 10.2.1.1](#)). [Table 4](#) should be used to summarize the data.

8.3.2 *Long Term Imprecision*

- (1) Long term imprecision requires the repeated use of reference material which is stable for at least several weeks in the laboratory. This test can only be performed if the manufacturer supplies such a material and is not appropriate for the reference method. An estimate of long term precision can then be made by tabulating the daily control results for the device, and analyzing the standard deviation and coefficient of variation for the cell types for which a reference material was repeatedly analyzed over a period of weeks.
- (2) An estimate of long term stability of imprecision can be made by comparing paired sample results gathered over a period of time. For example, a laboratory may wish to run four paired samples at the beginning of each week and calculate the imprecision. Longitudinal comparison of these weekly results would detect a change in precision. [Table 4](#) should be used to summarize the data.

TABLE 3

Log of Data for Comparing Distributional Inaccuracy*

	Specimen#	Total Leukocyte Count	Reference Lymphocyte Count (x)		$\frac{X_A + X_B^{**}}{4}$	Test Lymphocyte Count (y)***		$\frac{Y_C + Y_D}{2}$
			Slide A	Slide B	Mean (\bar{x})	C	D	Mean (\bar{y})
1								
2								
3								
4								
5								
6								
7								
98								
99								
100								

* Construct similar table for each cell type evaluated.

** 200 cell counts for X_A and X_B

*** If the test method is a flow system and a sample has been analyzed only once, enter the same result in both C and D.

TABLE 4

Short Term and Long Term Imprecision for Routine Samples

Cell Type	<u>Short term</u>		<u>Long Term</u>	
	Mean	CV	Mean	CV

NB: Similar tables are compiled for reference method, test method, (and routine method if desired).

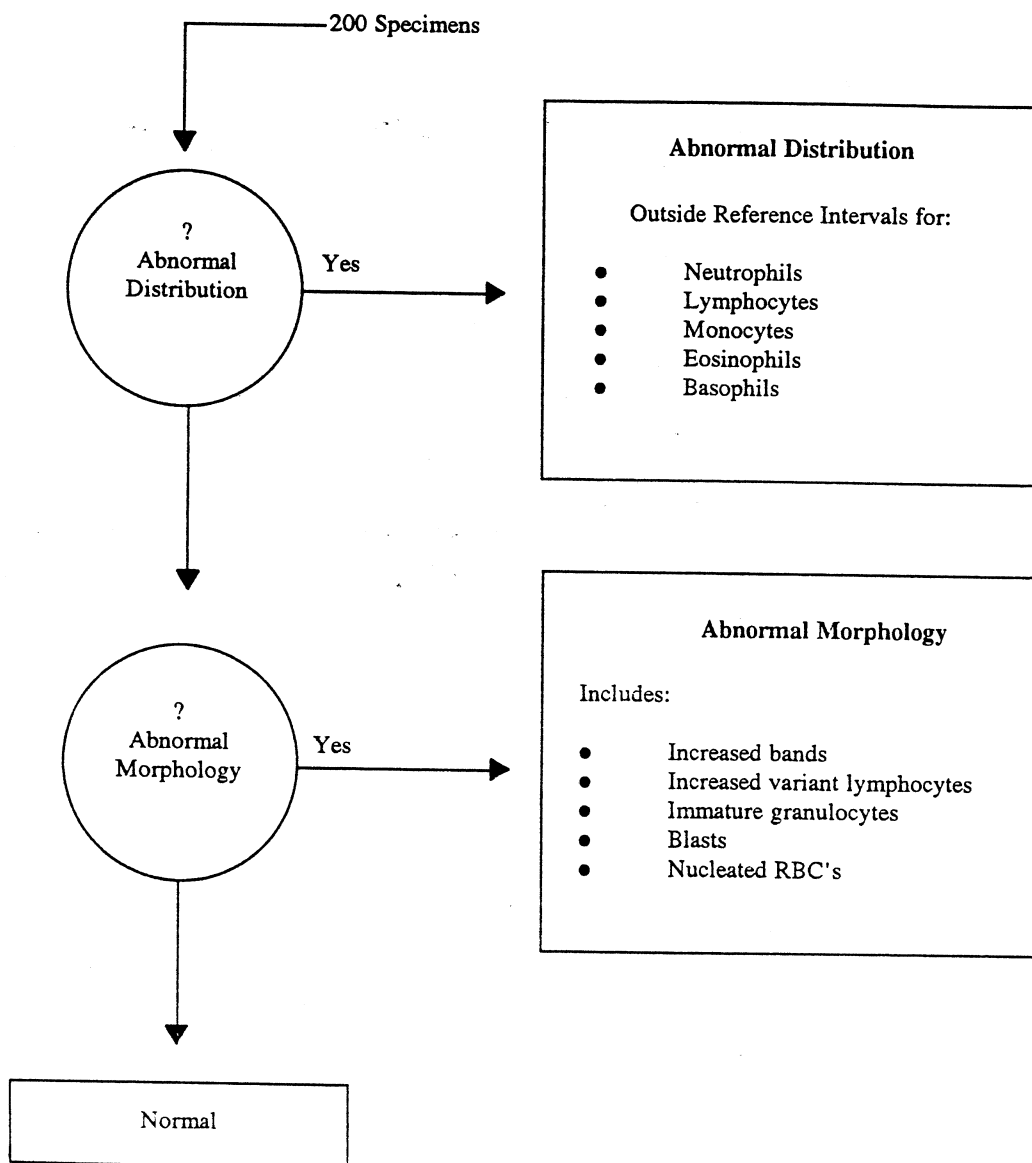
9.0 CLINICAL SENSITIVITY STUDY

There are three separate activities:

- (1) Development of reference (normal) values for both test and the reference method
- (2) Determination of sensitivity to finding of abnormal samples, (Figures 3 and 4)
- (3) Arbitration results by an arbitrator when reference and test methods disagree.

FIGURE 4

Outline of Clinical Sensitivity Study



9.1 Reference (Normal) Values

9.1.1 *Purpose*

The clinical validity of a method depends on reference to a set of normal values and is not dependent on exact agreement of reference and test method. If the two methods do not agree exactly, this protocol will nevertheless establish reference values for each method. These values are applied to determine clinical sensitivity, independent of any bias as compared to the reference method.

9.1.2 *Normal Subjects*

One hundred "normal" individuals are required for this part of the study. While it is recognized that a definition of normal is controversial, for purposes of this protocol, normal means an individual who has:

- (1) No clinical evidence of a medical disorder known to affect the differential leukocyte count
- (2) Had no recent episodes of upper respiratory infection
- (3) A blood count within the reference range for the other elements of the complete blood count (see [Table 5](#)), including the total leukocyte count,¹⁴ and has serum chemistry values, if available, that are not grossly abnormal.

TABLE 5
Hematology Reference Ranges

<u>Females*</u> %--tile	3-8 yrs		9-14 yrs**		15-64 yrs	
	5th	95th	5th	95th	5th	95th
hemoglobin (g/dL)	11.2	14.0	11.9	14.6	11.9	15.3
PCV (fraction)	0.329	0.403	0.348	0.424	0.353	0.448
red cells ($\times 10^{12}/L$)	3.9	5.0	4.0	5.0	3.9	5.1
white cells ($\times 10^9/L$)	4.9	11.9	4.8	11.0	4.5	11.1
<u>Males*</u>						
hemoglobin (g/dL)	11.2	13.9	12.1	15.0	13.6	16.9
PCV (fraction)	0.328	0.402	0.352	0.436	0.397	0.486
red cells ($\times 10^{12}/L$)	4.0	5.0	4.2	5.3	4.4	5.6
white cells ($\times 10^9/L$)	4.8	11.7	4.6	10.5	4.65	11.1

5th to 95th percentile range for hemoglobin concentration, packed cell volume (microhematocrit method), and red and white blood cell count by age and sex. Data from the National Health and Nutrition Examination Survey 1976-1980 (R. Fulwood et al. Vital and Health Statistics, Series 11, No. 232, 1982. DHHS Pub. No. (PHS) 83-1682. PHS, Washington, US Government Printing Office, 1982.)

*Measurements performed on 1000 females, 1100 males, 3 to 8 yrs old; 700 females, 750 males, 9 to 14 yrs old; 4700 females, 4500 males, 15 to 64 yrs old.

**For black females and males in this age group hemoglobin values are about 1 g/dL lower; PCV, 0.01 to 0.03 lower; red cell counts, 5th percentile about 10% lower; white cell counts, 5th percentile 10 to 20% lower, 95th percentile the same (males) to 10% lower (females).

Individuals who may qualify as normal, if they fulfill the above criteria, are laboratory personnel, outpatients coming for periodic (e.g., annual) checkups, and patients admitted for elective procedures. Blood donors are satisfactory normals if the sample is taken before the donation. Do not use the post-donation sample, which may be altered from baseline.

9.2 Sensitivity for Finding Abnormal Samples

9.2.1 *Rationale*

9.2.1.1 *Internal Sampling*

If an institution wishes to establish a new method, the test should be performed on samples available internally to give the best indication of usefulness within the institution. In general, the larger the sample series, the greater the confidence in the results.

9.2.1.2 *Scope of Samples*

We suggest that samples should be selected from the entire laboratory workload for a minimum of two concurrent weeks. This would include the types of samples encountered both in admitted patients and outpatients from a variety of clinics. In addition, the test method should be challenged against the most severe abnormalities the laboratory may encounter. It may be necessary to search out specific abnormal cases and process them by both methods.

Insert them at random as test samples. A list of specific sample types which should be included is given in [Table 2](#).

9.2.1.3 *Flagging System vs. Reference System*

The goal is to show how a system can identify abnormalities for further study by human observers (i.e., to flag) and to compare this ability to the reference system. The existing method may also be compared against the reference method at this time, using the same specimens. It is assumed in this method that all samples for which classifications agree between methods or systems have been successfully processed. Therefore, arbitration is limited to discrepant samples. ([See Section 10.3.3.](#))

The results of this entire procedure may be summarized as follows:

- (1) Samples in agreement
- (2) False positive ratio
- (3) False negative ratio.

10.0 DATA ANALYSIS

10.1 Comparison of Methods (Inaccuracy)

10.1.1 *Reference Method vs. Test Method*

The test for inaccuracy compares the two methods (i.e., the reference method and the test method). Consider either an automated or a routine laboratory method as the "test method." The aim is to determine whether differences in counts can be explained by the combined imprecision of the test and reference methods, or whether they represent true differences between the methods.

10.1.2 *Basis of Comparison*

10.1.2.1 *Statistical Basis*

The statistical basis of the comparison is a sample comparison of the means for each method for each cell type.

10.1.2.2 *Assumptions*

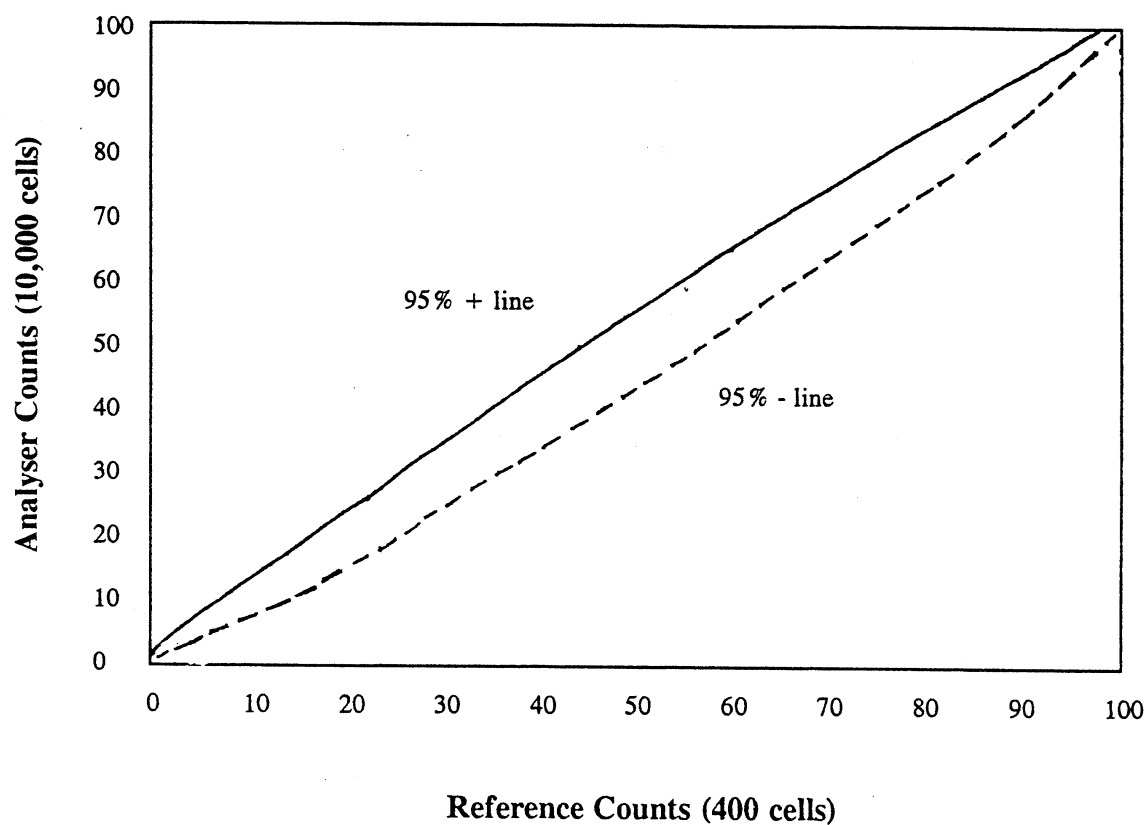
Assumptions for statistical analysis:

- (1) The reference method represents truth. (If accepted, then disagreements represent an inaccuracy of the test method.)
- (2) The test values (y) are Gaussian distributed at any given reference value (x).
- (3) The mean of the y values from each sample is a linear function of x .
- (4) This can be satisfactorily tested by examining the x - y scatterplot of the means of the reference method (x_i) versus the means of the test method (y_i) for each sample (i). On this scatterplot, superimpose the envelope that takes into account the imprecision of the test and reference methods. To simplify construction of the envelope, calculate the mean number of cells counted by the analyzer for all samples and insert this number as " n " (see [Figure 5](#)). Data points that fall outside the envelope limits should be examined for accuracy.

FIGURE 5

Reference Method Means vs. Test Method Means

10,000 vs. 400 Cell Count Envelope



— 95% + line
-- 95% -- line

10.1.2.3 *Formula Necessary to Examine for Inaccuracy*

$$SE = \left[\left(\frac{T}{n_t} \right) + \left(\frac{R}{n_r} \right) \right]^{\frac{1}{2}} \times 100$$

where:

T
is the estimated variance of the test method and,
 n_t

R
is the estimated variance of the reference method.
 n_r

$$T = p_t \times q_t$$

$$p_t = \frac{\text{mean value of } y_i}{100}$$

$$q_t = 1 - p_t$$

$$R = p_r \times q_r$$

$$p_r = \frac{\text{mean value of } x_i}{100}$$

$$q_r = 1 - p_r$$

n_t = total number of cells counted in both test analyses (C and D); or in C if only one test run was performed on that sample.

n_r = total number of cells counted in A and B (i.e., 400).

10.1.2.4 *Examination of Data*

Reference values within $\pm S_f \times SE$ are considered to be acceptable

where:

S_f = Student factor, which is 1.96 for 95% confidence.

10.1.3 *Variations*

In the analysis, variations in the data are accounted for as follows:

10.1.3.1 *Differences Between Specimens*

Differences between specimens (i.e., biologic variation) is assumed and accepted but not measured.

10.1.3.2 *Coherent Bias Between Methods*

Coherent bias between methods, if found significant, is determined by a difference in means between the two methods for each cell type.

10.1.3.3 *Nonrandom Interferences*

Known (nonrandom) interferences should have been segregated from these data, and therefore, results including known interferences should not influence the statistical results. For example, such an interference might occur in a patient with Pelger-Huët anomaly where conventional segmented neutrophils

are not present and the test method may disagree with the reference.

(NOTE: Such samples are not excluded from the analysis of clinical sensitivity).

10.2 Imprecision

10.2.1 *Methods*

Two methods to examine for imprecision are possible.

- (1) Short term imprecision
- (2) Long term imprecision.

Each test for imprecision is performed on both the reference and the test method.

10.2.1.1 *Short Term Imprecision*

Performance of short term imprecision studies as outlined in [Section 8.3](#) will yield differences between duplicates for each cell type.

The formula for short term standard deviation (SD_s):

$$SD_s = \left[\frac{\sum_{i=1}^n d_i^2}{2n} \right]^{\frac{1}{2}}$$

where:

n = number of samples

d_i = difference between duplicates for sample i

Calculate the short term coefficient of variation as

$$\frac{SD_s}{\text{Mean}} \times 100$$

10.2.1.2 *Long Term Imprecision*

Long term imprecision is calculated using the following formula for long term (SD_i) on control samples.

$$\text{Standard Deviation } SD_i = \left[\frac{\sum_{i=1}^n (x_i - \bar{x})^2}{n - 1} \right]^{\frac{1}{2}}$$

where:

n = number of samples

x_i = mean of results for day i

\bar{x} = mean of days or grand mean of all results

Calculate the long term coefficient of variation $\frac{SD_i}{\text{Mean}} \times 100$

10.2.2 *Presenting Results for Imprecision*

Use the format in [Table 4](#) for presenting results of the experiments for imprecision. Construct two such tables: one for the reference method and a second for the test method.

10.3 **Clinical Sensitivity**

10.3.1 *Reference Intervals*

- (1) Construct a histogram for each cell type from all normal values initially tabulated. Examine these histograms for outliers. These can be defined as more than >3 SD from the mean.
- (2) Recheck outliers to exclude transcription errors. If the outlier persists, remove it from the data set as not representative of normal. When an outlier is identified, remove its results from the whole file, including other cell types.
- (3) Derive the range of reference values from the histogram of the remaining values. Define the reference range as the central 95% of the population. Therefore, with 100 samples, exclude the highest and lowest two values, or a total of four. Tabulate these reference ranges by cell type for each method.

10.3.2 *Preliminary Classification of Clinical Sensitivity*

- (1) For each cell type, compare the mean of the two differential counts $[(A + B)/2]$ with the mean of the analyzer count, or the single analyzer count, if only one analysis has been performed.
- (2) Classify each study case into normal or abnormal for the test and reference methods. In addition, subdivide the abnormal cases as follows: those with abnormal proportions of one or more cell types (distributional or quantitative) using the reference intervals obtained in [Section 10.3.1](#); and those containing abnormal cells (morphological or qualitative) with or without abnormal proportions of normal cells. If only one observer has detected a morphological abnormality (e.g., a blast), this is classified as a

morphological abnormality by the reference method. Include "flags" generated by the analyzer for morphological abnormalities. Failure of the analyzer to provide results for one or more cell types is regarded as a morphological flag.

A study case may have both a distributional and a morphological abnormality. If the analyzer has failed to give differential count results on a sample, then exclude that sample from distributional analysis while including it as a morphological abnormality. Keep a list of all samples excluded from distributional analysis.

Prepare two matrix tables (distributional and morphological) which summarize these preliminary classifications ([Tables 6 and 7](#)).

TABLE 6

Preliminary Distributional Classification

		Results of Test Method	
		Positive (Abnormal)	Negative (Normal)
Reference Method	Positive (Abnormal)	TP (True Positive)	FN (False Negative)
	Negative (Normal)	FP (False Positive)	TN (True Negative)

Summary

$$\text{Agreement} = \frac{\text{TP} + \text{TN}}{\text{TP} + \text{FN} + \text{FP} + \text{TN}} \times 100 = \%$$

$$\text{False Positive Ratio} = \frac{\text{FP}}{\text{FP} + \text{TN}} \times 100 = \%$$

$$\text{False Negative Ratio} = \frac{\text{FN}}{\text{FN} + \text{TP}} \times 100 = \%$$

TABLE 7

Preliminary Morphological Classification

		Results of Test Method	
		Positive (Abnormal)	Negative (Normal)
Reference Method	Positive (Abnormal)	TP (True Positive)	FN (False Negative)
	Negative (Normal)	FP (False Positive)	TN (True Negative)

Summary

$$\text{Agreement} = \frac{\text{TP} + \text{TN}}{\text{TP} + \text{FN} + \text{FP} + \text{TN}} \times 100 = \%$$

$$\text{False Positive Ratio} = \frac{\text{FP}}{\text{FP} + \text{TN}} \times 100 = \%$$

$$\text{False Negative Ratio} = \frac{\text{FN}}{\text{FN} + \text{TP}} \times 100 = \%$$

- (3) Prepare a listing of all specimens showing disagreements, and subject these cases to the arbitration method outlined in [Section 10.3.4](#).

10.3.3 Arbitration**10.3.3.1 Limitations**

Arbitration is limited to samples with discrepant results between reference and test methods for distributional or morphological findings. The arbitrator is a qualified examiner, frequently with additional expertise and experience (see Section 4.2), who has not performed any of the reference differential counts heretofore in the study.

10.3.3.2 Method of Arbitrating

- (1) For all discrepancies between reference and test methods, check that the films are well made and adequately stained, and that there are no transcription errors.
- (2) For morphological disagreements:
 - (a) Where the abnormality has been identified in both reference slides, check the analyzer results to confirm that no morphological flags were triggered.

- (b) Where only one examiner identified an abnormality, scan both slides to confirm the morphological abnormality in one or both slides.
 - (c) Where an abnormality was flagged by the analyzer but neither examiner found a morphological abnormality, scan both slides carefully to ensure that no abnormality was missed by both examiners.
- (3) For distributional disagreements:
- (a) Review the duplicate analyses by the reference method (and the duplicate analyses by the test method if available) for each sample to determine whether the difference between duplicate results for each analysis was greater than would be expected due to imprecision of the method, using the approach for qualification of examiners given in [Section 5.6.3](#).
 - (b) If the difference between the reference counts is greater than would be expected, perform a 200-cell differential count and compare the results with those of the original counts. If the arbitrator's count agrees with one of the two original counts within expected limits using Standard Error of Proportion, but not with the other, then replace that count with the arbitrator's count and reassess clinical sensitivity for that sample. If the arbitrator's count agrees with both original counts (i.e., it is between the two original counts) then use the original counts.
 - (c) If the difference between the original reference counts is within expected limits, no arbitration is necessary.
 - (d) If the difference between test method results is greater than would be expected for the number of cells counted, check the analyzer result output to ensure that no malfunction flag was triggered due to a sample abnormality which might invalidate the differential count results and cause that sample to be excluded from distributional analysis while being classified as a test method morphological abnormality.

10.3.3.3

Revisions

Revise the main tables of classifications using the arbitration findings. Call the new tables "Final Distributional/ Morphological Classification." ([See Tables 8 and 9.](#))

TABLE 8**Final Distributional Classification**

		Results of Test Method	
		Positive (Abnormal)	Negative (Normal)
Reference Method	Positive (Abnormal)	TP (True Positive)	FN (False Negative)
	Negative (Normal)	FP (False Positive)	TN (True Negative)

Summary

$$\text{Agreement} = \frac{TP + TN}{TP + FN + FP + TN} \times 100 = \%$$

$$\text{False Positive Ratio} = \frac{FP}{FP + TN} \times 100 = \%$$

$$\text{False Negative Ratio} = \frac{FN}{FN + TP} \times 100 = \%$$

TABLE 9**Final Morphological Classification**

		Results of Test Method	
		Positive (Abnormal)	Negative (Normal)
Reference Method	Positive (Abnormal)	TP (True Positive)	FN (False Negative)
	Negative (Normal)	FP (False Positive)	TN (True Negative)

Summary

$$\text{Agreement} = \frac{TP + TN}{TP + FN + FP + TN} \times 100 = \%$$

$$\text{False Positive Ratio} = \frac{FP}{FP + TN} \times 100 = \%$$

$$\text{False Negative Ratio} = \frac{FN}{FN + TP} \times 100 = \%$$

10.3.3.4 *Disagreement*

As a supplement to the tables, prepare a list of the remaining samples with disagreement. Include the sample number and the cause of disagreement (e.g., "Sample #63 blast cells in reference missed in test").

10.3.4 *Sensitivity to Finding Abnormals*

Classify samples as distributional and/or morphological abnormalities, and calculate the false positive and false negative ratios as described in Sections 4.7 and 4.8. Some cases may have both distributional (one or more) as well as morphological abnormalities.

10.3.5 *Interpreting Results*

10.3.5.1 *Patterns*

It is expected that results will follow certain well-established patterns. For example, discrepancies of distributional abnormalities can be expected because 95% limits were used to establish the normal range. Normal samples randomly exceeding the central 95% of samples may not be the same patients in both methods.

The number of such "chance discrepancies" will depend on the number of tested parameters and on the precision of the reference and test methods.

10.3.5.2 *Low Concentration Samples*

Similarly there is a defined probability for discovering any abnormal cell type when present in low concentration. It is expected that an equal number of false negatives would exist in the reference and test methods performed on equal numbers of cells. However, accessory information regarding the sample frequently influences technologists to look for certain abnormal cell types by scanning the slide. The study must either ensure that these data are excluded from the tabulated results, or must accept a bias in performance which is not completely predictable.

If such a bias exists, resolve it by further arbitration under strictly controlled conditions.

10.3.5.3 *Application of Analyses*

Because sample selection includes both routine laboratory samples and additional selected samples for specific conditions, the analyses may be performed on the entire population of samples or on a subset representing a group of selected samples. [The performance of a number of differential leukocyte counters when analyzed using an earlier version of this document (H20-T) has been published.¹⁵⁻²⁰]

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**SUMMARY OF COMMENTS ON H20-T, LEUKOCYTE DIFFERENTIAL
COUNTING; Tentative Standard**

NOTE: The section identifiers in the comments refer to H20-T and responses, unless indicated otherwise, refer to H20-A.

GENERAL

1. The term "expert" or "panel of experts" is used throughout the document without any attempt to define it.
 - **The "qualified examiner" is defined in Section 4.0(17) of H20-A. The individual examiners are qualified by the procedure outlined in Section 5.6.4.**
2. Why can't spun films be an acceptable reference method? These slides are of superior quality morphologically. How does one account for cell "normal" values when spun is referenced to wedge?
 - **Although spun films are satisfactory in many ways, the problems associated with aerosolization of blood during the preparation were felt to be significant. Therefore the committee chose one reference method. See Universal Precautions (NCCLS M29-T2).**
3. I believe the document would be greatly improved and have a greater likelihood of being used if there were detailed examples of each of the calculations used. The rather complex and unfamiliar mathematical and statistical treatment required to apply the standard will diminish its use in laboratories.
 - **The complex and confusing statistical methods have been deleted and the revised document employs standard methods only.**

SPECIFIC

FOREWORD

4. I disagree with the statement in the Foreword that reporting in proportional concentrations be accepted for the time being. Several individuals and bodies regard absolute leukocyte differential counts as the preferred reporting method. At least one system on the market performs primarily absolute counts and also proportional concentrations as well. Additionally, there could possibly be hitherto unrecognized biases between methods which may be revealed. I recommend that all calculations be made for absolute differential leukocyte counts as well as for proportional counts, and the relevant portions of the standard should be changed appropriately.
 - **Although the committee is in agreement, the problems with developing accurate absolute count using the manual method precluded this method. (Also see the Foreword of H20-A.)**

Section 1.1

5. Line 4: Suggest deletion of the phrase "and accuracy". The predetermined criteria of automated instruments do not automatically improve accuracy. An NCCLS Standard should not confuse these basic laboratory concepts of precision and accuracy.

- **This has been done.**

6. Line 5: Suggest replacing the phrase "multiple hundreds of cells" with "many more cells than can be conveniently or comfortably classified by human visual examination."

- **This has been done.**

Section 1.2.1

7. This paragraph fails to recognize two other automated instrument techniques for differential counting, namely impedance counters (e.g., - Coulter) or dual-angle laser instruments (e.g., - Ortho ELT-8/ws).

- **The subcommittee feels that all presently marketed automated differential counters are covered in this section. Both instruments mentioned are flow systems.**

Section 4.0

8. The last sentence in the definition of Reference Method should read, "This imprecision can be reduced...", not minimized. Minimization is a well defined concept, and to minimize the imprecision would take many replicates, not two.

- **This has been done.**

Section 4.12

9. I wonder if the definition of a "routine examiner" needs to be further specified as one who has specific experience in peripheral blood morphology.

- **This is certainly implied in the definition of "routine examiner" and "routine laboratory method." (See Section 4.0 (17) and (19) of H20-A.)**

Section 4.13

10. Line 3: "staining" does not apply to all automated systems (see above comment on 1.2.1) and should be followed by "(where applicable)."

- **The section has been modified to avoid the confusion. "Staining" is no longer included as a potential part of the system.**

Section 5.1.1

11. During my recent review of NCCLS approved Standard H7-A (Procedure for Determining Packed Cell Volume by the Microhematocrit Method), I noted that tripotassium EDTA was prohibited as the anticoagulant because of its cell shrinkage effect as compared to disodium EDTA. The present standard advocates tripotassium EDTA without reference to disodium EDTA or why the former must be used. Clarification would be in order.

- **We have chosen to continue recommending K_3EDTA due to its widespread use. Morphology is preserved. NA_2EDTA is only required when calibrating these instruments.**

Section 5.1.5.1(4)

12. If the thread-like filaments do not contain chromatin, what do they contain? If the word "chromatin" is used in the hematologic sense of dark or condensed nuclear material (as opposed to lighter, less dense "parachromatin") then the filament is chromatin. If "chromatin" is used in the more general cell biology sense of nuclear contents, then the filaments do contain chromatin, because they have nuclear material between the opposing segments of nuclear envelope. Suggest that this sentence end with "filaments."

- **The subcommittee agrees. The suggested change has been made.**

Section 5.1.5.2

13. Suggest that "stab" be added as another common term for this cell type.

- **This has been done.**

Section 5.1.5.3(7)

14. Line 4: While I understand the intent of the authors, I cannot find the word "chroma" in my medical dictionary.

- **This was a typographical error which has been corrected.**

Section 5.1.6.2(2)

15. I suggest adding the sentence "The total leukocyte count of the sample should be determined by an acceptable method, and the result recorded."

- **The subcommittee agrees. In order to avoid confusion a phrase noting that the result be recorded has been added (CF. 8.1.7).**

Section 5.1.6.2(4)

16. The eosinophil is usually the only cell which, when disrupted, is still recognizable as such because of its bright orange refractile granules. All disrupted cells should be classified as "other" to avoid artificially increasing the proportion of eosinophils in a film with many degenerate forms.

- **Other degenerated leukocytes may also be recognizable and also would be recorded. The eosinophil is given as an example and is not meant to exclude other cell types which may be identifiable.**

Section 5.1.6.3

17. I find no Figure 1 that is appropriate for this discussion. They may be referring to the classifications in Table 1.

- **The revised version has corrected this oversight.**

Section 5.1.6.3(3)

18. Delete period and continue sentence as: "and as absolute numbers of each cell type."

- **Although the subcommittee agrees with the basic notion being advanced, the present protocol does not require absolute counts. Hopefully, future revisions will use absolute numbers only.**

Section 7.6.2

19. Is "mid-range sample" a term that is generally understood?

- **In the revision this term is no longer used.**

Section 8.1.2

20. I question the use of five separate categories: "Sample in agreement, Test false abnormal, test false normals, Reference false abnormal, and Reference false normals." As these are discrepant sample results between two methods, in four cases, the one category is similar to the other, but just receives a different name. For instance, "Test false abnormal" is equivalent to "Reference false normals." However, if it was intended that the reference method be arbitrated as well, as is implied later in the document, this should be stated clearly early in the document.

- **The revision should make this point more understandable.**

Section 8.3

21. As the test method may or may not use a slide, the words "or run" should be inserted wherever a reference is made to the slide of the test method. Thus, in paragraph 8.3.1, the sentence should read: "For purposes of this study, only the first run or slide of each method is compared. That is, slide A is compared to slide or run C." In paragraph 8.3.5, the sentence should read: "Slide or run B and slide D are now classified..." In paragraph 8.3.6, the sentence should conclude: "...another matrix table comparing slide or run C with slide or run D."

- **These additions have been made in the document.**

Section 8.4.4

22. "Significant numbers of Disagreements" is not defined (perhaps, deliberately).

- **The present document primarily outlines the method of analysis. Section 10.3.3 of H20-A outlines expected rates of discrepancy.**

Section 9.1.2.5

23. (1) Should indicate here that X values are the reference method results.

- **This has been added to the revised document.**

24. (3) and (4) The F-test that is used for testing Row, Column, and Interaction effects does assume these things, even though we know them to be incorrect assumptions. I don't think they've modified the ANOVA to account for this (nor should they). Perhaps, what the authors mean is that we need to be aware of the failure to meet the ANOVA assumptions, and to take this into account when we interpret the results.

- **The revised statistical analysis has dropped the F-test as a method of comparison.**

Section 9.1.4

25. They describe a nifty technique for removing the interaction. However, they might check (if they haven't) on using corrected slope, which takes into account the error in the reference values. See an article by Cornbleet and Gochman, "Incorrect Least-Squares Regression Coefficients in Method Comparison Analysis" *Clinical Chemistry* 25(3): 432-438 (1979).

- **This section has been completely revised and the slopes are no longer determined.**

Section 9.2.2

26. Are these ANOVA runs on raw or transformed data? Also, the CV in their formula should be multiplied by 100%.

- **No longer included in revised document.**

Section 9.2.3

27. Between-run imprecision will contain variability due to within-run imprecision, because of the design where a single sample is run on 10 different days. If, instead, the design called for duplicate specimens on each of the 10 days, rather than 10 on one day and one on each of 10 more days, there would be unified estimates of within and between-run imprecision. As a by-product of this design, the Mean Square for Error in the ANOVA would be the "average variation" needed in Section 9.2.5.2.

- **The imprecision analyses have been revised and simplified.**

Section 9.2.5

28. Sample Size Efficiency could be defined and motivated better. Why do we need it? What is good efficiency?

- **This term is no longer included in the document.**

Section 9.2.5.2

29. (2) $X(K)$ should be X_K in this formula. Also, this K is different from K in Section 9.1.2.2; the notation should be consistent.

- **The revised document no longer employs this calculation.**

- (4) Q should be defined.

- **No longer included.**

Section 9.3.1

30. The outlier process seems too strict. Since it is possible to visually examine all data, the only outlier screen necessary is to remove those values that are so extreme they were obviously caused by technical or clerical error, or by extreme machine malfunction. The 3 SD screen will remove valid data points and artificially reduce the observed variation.

- **A new method for outlier identification has been included in the revision.**

TABLE 4

31. The letters a b c d etc., are not explained in Section 4.3.2, which is nonexistent.

- **The tables have been completely revised and these symbols are no longer used.**

TABLES 8 and 9

32. It is not clear what the letters a - i stand for.

- **See above comment.**

TABLES 15 to 19

33. Should be presented as conventional ANOVA tables, with proper labeling of the row and column factors, with standard p-values (not 1-p), and with the usual interpretative statistics: the Mean Square for Error and R-Square. It is very difficult to make much sense of the output from an obscure and highly unconventional program.

This example could be quite useful if the authors used it to demonstrate the entire protocol, including filling in Tables 2 through 9. This would, perhaps, be more useful than listing all that data.

- **The revised document has eliminated these tables. These analytical methods are no longer included.**

FIGURES

34. I believe it would make the document more general if the scattergram examples merely referred to a pattern recognition counter in some other way that did not specifically identify a particular product.

- **The revised document has eliminated any reference to specific instruments.**

RELATED NCCLS PUBLICATIONS

- EP5-T2** **USER EVALUATION OF PRECISION PERFORMANCE OF CLINICAL CHEMISTRY DEVICES—Second Edition; Tentative Guideline (1992).** Detailed procedure for the user to establish the performance characteristics of *in vitro* diagnostic devices in the user's laboratory.
Chairholder: John W. Kennedy
Medstat Consultants
- EP10-T** **PRELIMINARY EVALUATION OF CLINICAL CHEMISTRY METHODS; Tentative Guideline (1989).** Experimental design and data analysis for preliminary evaluation of the performance of an analytical method or device.
Chairholder: Richard B. Passey, Ph.D.
University of Oklahoma
- GP10-P** **ASSESSMENT OF CLINICAL SENSITIVITY AND SPECIFICITY OF LABORATORY TESTS; Proposed Guideline (1987).** Design of a study to evaluate the clinical accuracy of laboratory tests; procedures for preparing ROC curves; and glossary of terms.
Chairholder: Mark H. Zweig, M.D.
National Institutes of Health
- H1-A3** **EVACUATED TUBES FOR BLOOD SPECIMEN COLLECTION—Third Edition; Approved Standard (1991).** Requirements for blood collection tubes and additives.
Chairholder: Harald Jacoby
- H3-A3** **PROCEDURES FOR THE COLLECTION OF DIAGNOSTIC BLOOD SPECIMENS BY VENIPUNCTURE—Third Edition; Approved Standard (1991).** Methods for the collection of blood specimens by venipuncture and appropriate training program aimed at increasing analyte integrity and minimizing laboratory error. Includes a 24-step protocol for specimen collection, recommendations for "order of draw" and considerations for performing venipuncture on children.
Chairholder: Harald Jacoby
- H4-A3** **PROCEDURES FOR THE COLLECTION OF DIAGNOSTIC BLOOD SPECIMENS BY SKIN PUNCTURE—Third Edition; Approved Standard (1991).** Detailed description and explanation of proper collection and hazards to patients due to inappropriate specimen collection by skin puncture procedures.
Chairholder: Harald Jacoby
- H18-A** **PROCEDURES FOR THE HANDLING AND PROCESSING OF BLOOD SPECIMENS; Approved Guideline (1990).** Addresses the multiple factors associated with handling and processing specimens, factors that can introduce imprecision or systematic bias into test results.
Chairholder: Roger R. Calam, Ph.D.
St. John Medical Center, Detroit

- H26-P** **PERFORMANCE GOALS FOR THE INTERNAL QUALITY CONTROL OF MULTICHANNEL HEMATOLOGY ANALYZERS; Proposed Standard (1989).** Recommended performance goals for analytical accuracy and precision based on mathematical models for the following measurements: hemoglobin concentration, erythrocyte count, leukocyte count, platelet count, and mean corpuscular volume.
Chairholder: Michael B. O'Sullivan, M.D.
 Mayo Clinic
- H35-P** **ADDITIVES TO BLOOD COLLECTION DEVICES: EDTA; Proposed Standard (1989).** Technical description of ethylenediaminetetraacetic acid (EDTA) and its use in blood collection products.
Chairholder: Erwin Coyne, Ph.D.
 Loyola University Medical Center
- M29-T2** **PROTECTION OF LABORATORY WORKERS FROM INFECTIOUS DISEASE TRANSMITTED BY BLOOD, BODY FLUIDS, AND TISSUE — Second Edition; Tentative Guideline (1991).** Guidance on the risk of transmission of hepatitis B virus and human immunodeficiency viruses in the laboratory; specific precautions for preventing transmission of bloodborne infection during clinical and anatomical laboratory procedures.
Chairholder: Stanley Bauer, M.D.
 Beth Israel Medical Center