
Histochemical Method for Leukocyte Alkaline Phosphatase

* This document is no longer being reviewed as part of the NCCLS consensus process. However, because of its usefulness to a limited segment of the clinical laboratory community, NCCLS is continuing to make the document available for its informational content.

Procedure for a semi-quantitative assay for determining leukocyte alkaline phosphatase; criteria for scoring the assay and interpreting the results.



This NCCLS document was published before Centers for Disease Control publication of universal precautions for the prevention of transmission of infectious disease. Universal precautions should be observed when handling specimens from all patients. See NCCLS M29-T2 *Protection of Laboratory Workers from Infectious Disease Transmitted by Blood, Body Fluids, and Tissue; Second Edition; Tentative Guideline.* (Vol. 11 No. 14)



NCCLS...

Serving the World's Medical Science Community Through Voluntary Consensus

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

In addition to developing and promoting the use of voluntary consensus standards and guidelines, NCCLS provides an open and unbiased forum to address critical issues affecting the quality of patient testing and health care.

PUBLICATIONS

An NCCLS document is published as a standard, guideline, or committee report.

Standard A document developed through the consensus process that clearly identifies specific, essential requirements for materials, methods, or practices for use in an unmodified form. A standard may, in addition, contain discretionary elements, which are clearly identified.

Guideline A document developed through the consensus process describing criteria for a general operating practice, procedure, or material for voluntary use. A guideline may be used as written or modified by the user to fit specific needs.

Report A document that has not been subjected to consensus review and is released by the Board of Directors.

CONSENSUS PROCESS

The NCCLS voluntary consensus process is a protocol establishing formal criteria for:

- The authorization of a project
- The development and open review of documents
- The revision of documents in response to comments by users
- The acceptance of a document as a consensus standard or guideline.

Most NCCLS documents are subject to two levels of consensus—"proposed" and "approved." Depending on the need for field evaluation or data collection, documents may also be made available for review at an intermediate (i.e., "tentative") consensus level.

Proposed An NCCLS consensus document undergoes the first stage of review by the healthcare community as a proposed

standard or guideline. The document should receive a wide and thorough technical review, including an overall review of its scope, approach, and utility, and a line-by-line review of its technical and editorial content.

Tentative A tentative standard or guideline is made available for review and comment only when a recommended method has a well-defined need for a field evaluation or when a recommended protocol requires that specific data be collected. It should be reviewed to ensure its utility.

Approved An approved standard or guideline has achieved consensus within the healthcare community. It should be reviewed to assess the utility of the final document, to ensure attainment of consensus (i.e., that comments on earlier versions have been satisfactorily addressed), and to identify the need for additional consensus documents.

NCCLS standards and guidelines represent a consensus opinion on good practices and reflect the substantial agreement by materially affected, competent, and interested parties obtained by following NCCLS's established consensus procedures. Provisions in NCCLS standards and guidelines may be more or less stringent than applicable regulations. Consequently, conformance to this voluntary consensus document does not relieve the user of responsibility for compliance with applicable regulations.

COMMENTS

The comments of users are essential to the consensus process. Anyone may submit a comment, and all comments are addressed, according to the consensus process, by the NCCLS committee that wrote the document. All comments, including those that result in a change to the document when published at the next consensus level and those that do not result in a change, are responded to by the committee in an appendix to the document. Readers are strongly encouraged to comment in any form and at any time on any NCCLS document. Address comments to the NCCLS Executive Offices, 940 West Valley Road, Suite 1400, Wayne, PA 19087, USA.

VOLUNTEER PARTICIPATION

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

Histochemical Method for Leukocyte Alkaline Phosphatase

Message to the User

This proposed standard describes the semi-quantitative, histochemical method for determining leukocyte alkaline phosphatase (LAP). The method includes procedures for preparing fixative, buffer, and counterstains; for staining blood smears; scoring criteria for determining LAP activity; and guidance for interpreting the LAP activity score.

The subcommittee hopes that this proposed standard method will improve the reliability and interpretation of this clinically useful, widely used test.

This document is the result of much effort by the members of the Subcommittee on Cellular Enzymology. Now, we look to our membership--the clinical laboratory community--to participate in the consensus process. A questionnaire is appended to this document so that you can give us your views on the value of this project and its execution as represented by this standard. A proposed standard is the first stage in the NCCLS consensus process, and it is, therefore, especially important that we have your responses to the first three questions relating to the document's scope, utility, and scientific validity.

Your comments will help ensure that the document reflects your needs as a member of the clinical laboratory community and will help the subcommittee in related efforts in the future.

*Kouichi R. Tanaka, M.D., Chairholder
Subcommittee on Cellular Enzymology*

October 1984

Histochemical Method for Leukocyte Alkaline Phosphatase

VOLUME 4 NUMBER 14

Kouichi R. Tanaka, M.D.
Ernest Beutler, M.D.
Leonard S. Kaplow, M.D.
Robert E. Plapinger, Ph.D.
Akira Yoshida, Ph.D.

© 1984. The National Committee for Clinical Laboratory Standards. All rights reserved. This publication, or parts thereof, may not be reproduced in any form without permission of NCCLS.

Approved by Board of Directors as:

Proposed Standard

December 1983

Published:

October 1984

TABLE OF CONTENTS

	<u>PAGE</u>
MESSAGE TO THE USER	i
COMMITTEE MEMBERSHIP	vi
FOREWORD	vii
1.0 PURPOSE	1
2.0 PRINCIPLE	1
3.0 APPARATUS	1
4.0 REAGENTS	1
5.0 SOLUTIONS	2
6.0 STAINING PROCEDURE	2
7.0 SCORING PROCEDURE	3
8.0 INTERPRETATION OF RESULTS	4
9.0 COMMENTS	5
REFERENCES	6
RELATED NCCLS PUBLICATION	7

AREA COMMITTEE ON HEMATOLOGY

Robert V. Pierre, M.D.
Chairholder

Mayo Clinic Center
Rochester, Minnesota

SUBCOMMITTEE ON CELLULAR ENZYMOLOGY

Kouichi R. Tanaka, M.D.
Chairholder

Harbor-UCLA Medical Center
Torrance, California

Ernest Beutler, M.D.

Scripps Clinic & Research Foundation
La Jolla, California

Leonard S. Kaplow, M.D.

Veterans Administration Hospital
West Haven, Connecticut

Robert E. Plapinger, Ph.D.

FDA Center for Devices and Radiological
Health
Rockville, Maryland

Akira Yoshida, Ph.D.

City of Hope National Medical Center
Durate, California
St. Louis, Missouri

Onno W. van Assendelft, M.D., Ph.D.

Centers for Disease Control
Atlanta, Georgia

Mary G. Hoffman
Staff Liaison

NCCLS
Villanova, Pennsylvania

FOREWORD

Neutrophilic leukocytes contain an alkaline phosphomonoesterase with variable activity in normal subjects and in patients with hematological and nonhematological disorders. Leukocyte alkaline phosphatase is a well established term and thus will be used, although we recognize that in the peripheral blood the enzyme is virtually restricted to neutrophil polymorphonuclear cells.

There is no relationship between leukocyte alkaline phosphatase and serum alkaline phosphatase. The function of leukocyte alkaline phosphatase is still unknown, but normal leukocyte alkaline phosphatase depends on an intact pituitary adrenal axis.

Clinical interest in leukocyte alkaline phosphatase was stimulated initially by the observation that the leukocytes of patients with chronic granulocytic leukemia usually had low levels of enzymatic activity in contrast to high values seen in leukocytosis of other disorders. Quantitative methods of assay are available but are not practical. Semiquantitative assay by a variety of azo dye histochemical methods has made determination of leukocyte alkaline phosphatase a widely used clinical test. This document describes a standard histochemical method which should serve as a benchmark.

KEY WORDS

Leukocyte alkaline phosphatase (LAP); phosphatase activity; LAP, histochemical method; granulocytic leukemia; leukocytosis; LAP, scoring criteria.

HISTOCHEMICAL METHOD FOR LEUKOCYTE ALKALINE PHOSPHATASE

1.0 PURPOSE

Estimating leukocyte alkaline phosphatase activity is most useful clinically in differentiating chronic granulocytic leukemia from leukocytosis as seen in severe infections, polycythemia rubra vera, and myelofibrosis with myeloid metaplasia.

2.0 PRINCIPLE

The substrate is hydrolysed by enzymatic activity at pH 9.5 releasing phosphate and an aryl naphtholamide. The aryl naphtholamide immediately couples with the diazonium salt present in the incubation mixture forming an insoluble azo dye at the theoretical sites of enzyme activity.

3.0 APPARATUS

Routinely used, clean glassware

4.0 REAGENTS

- (1) naphthol AS-BI phosphate, sodium salt
- (2) fast violet B salt (purified), 6-benzamido-4-methoxy-m-touidine, diazonium chloride
- (3) 2-amino-2-methyl-1,3-propanediol
- (4) 37% formaldehyde
- (5) absolute methanol
- (6) absolute acetone
- (7) 0.1 N hydrochloric acid
- (8) hematoxylin powder
- (9) sodium iodate
- (10) aluminum potassium sulfate, $\text{AlK}(\text{SO}_4)_2 \cdot 12 \text{H}_2\text{O}$
- (11) 0.03 M sodium citrate, $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2 \text{H}_2\text{O}$ - (4.41 g/500 mL H_2O)

(12) 0.03 M citric acid H₂O - 3.15 g/500 mL H₂O

5.0 SOLUTIONS

5.1 Fixative

Sixty percent acetone buffered to pH 4.2 to 4.5 with 0.03 M citrate buffer. Add 168 mL of 0.03 M citric acid to 32 mL of 0.03 M sodium citrate and then add slowly, while stirring, 300 mL of absolute acetone. Store at room temperature.

5.2 Buffer

5.2.1 Stock--0.2 M propanediol. Dissolve 21 g of 2-amino-2 methyl-1, 3-propanediol in distilled water and dilute to 1000 mL with distilled water. Store in the refrigerator.

5.2.2 Working--0.05 M propanediol, pH 9.4 to 9.6. To 250 mL of stock propanediol, add 70 mL of 0.1 N HC1 and dilute to 1000 mL with distilled water. Store in refrigerator, but warm to room temperature before use.

5.3 Counterstain

5.3.1 Add 1 g of hematoxylin to 500 mL distilled water.

5.3.2 Heat to the boiling point and dilute to 900 mL with distilled water.

5.3.3 Add 0.2 g of sodium iodate and 50 g of aluminum potassium sulfate, and shake the mixture well.

5.3.4 Dilute to 1000 mL with distilled water and store in a brown bottle at room temperature. Counterstain remains stable for 3 to 4 months.

5.3.5 Filter just prior to use.

5.3.6 Instead of the above preparation, a working solution of reagents (4), (8), (9), and (10) is available as Mayer's hematoxylin.

6.0 STAINING PROCEDURE

6.1 Use fresh, thin smears of peripheral blood. If venous blood is drawn, anticoagulate with heparin, (Do not use EDTA.) If staining will be

delayed longer than 12 h after blood collection, fix smears and store in a freezer during the interim.

- 6.2 Fix blood smears 10 seconds at room temperature in 60% acetone citrate, pH 4.2.
- 6.3 Wash in slowly running tap water for 30 to 60 seconds.
- 6.4 Carefully air dry or blot dry.
- 6.5 Place dried slides for exactly 15 min at room temperature in freshly prepared substrate mixture in a Coplin jar. Prepare the substrate mixture by placing about 5 mg of naphthol AS-BI-PO₄ in a dry 125 mL Erlenmeyer flask to which 60 ML of 0.05 M working propanediol buffer (pH 9.4 to 9.6) and approximately 40 mg of diazonium salt are added. Shake well, and filter into a Coplin jar; use immediately after preparation.

7.0 **SCORING PROCEDURE**

- 7.1 Scan the smear (~450x) and select an are where the erythrocytes are barely touching one another. The sites of phosphates activity are represented by granulation in the cytoplasm varying from pale pink to red. The counterstained nuclei appear blue gray (see 9.4).
- 7.2 Using the oil immersion lens, rate 100 consecutive segmented neutrophilic leukocytes from zero to four plus on the basis of the intensity of the precipitated dye. The criteria for scoring are summarized in the table. The sum of the 100 ratings is considered the score for a particular smear. Thus, scores may range from a minimum of zero to a maximum of 400. It is important to score only polymorphonuclear leukocytes. Do not include lymphocytes, monocytes, basophilis, and eosinophils in the score.

TABLE
SCORING CRITERIA

Precipitated Azo Dye in Cytoplasm			
Cell Rating	Amount ^a (%)	Size of Granule	Intensity of Staining
0	None	-----	None
1 +	< 50	Small	Faint
2 +	50-80	Small to medium	Moderate
3 +	80-100	Medium to large	Strong
4 +	100	Medium and large	Brilliant

^aPercentage of volume of cytoplasm occupied by azo dye precipitate.

8.0 INTERPRETATION OF RESULTS

- 8.1 In humans, alkaline phosphatase activity is found only in some mature and band form neutrophils, and in a very rare lymphocyte (weak reaction). All other cells of the peripheral blood are negative.
- 8.2 The normal human has little neutrophil alkaline phosphatase, and the normal average score is 60. The range of normal scores is wide, varying from 15 to 100. Each laboratory should establish its normal range.
- 8.3 Increased activity is seen, for example, in infectious leukocytoses, polycythemia vera, pregnancy or use of oral contraceptives, and most cases of multiple myeloma and active Hodgkin's disease.
- 8.4 Abnormally low or absent staining is seen in chronic granulocytic leukemia, paroxysmal nocturnal hemoglobinuria, and hereditary hypophosphatasia.
- 8.5 The leukocyte alkaline phosphatase activity score is not diagnostic of any disease.

9.0 COMMENTS

- 9.1 Store the diazonium and naphthol salts in the freezer, preferably in a dessicator. The diazonium salts are potentially carcinogenic and should be handled with care. Avoid contact with skin or inhalation.
- 9.2 If the diazonium salt does not dissolve in the aqueous buffer solution, it has probably undergone decomposition; check its "coupling power" at alkaline pH with naphthol AS-BI or B naphthol.
- 9.3 In scoring, select areas on the slide where the erythrocytes are relatively thinly spread out and barely touch or overlap one another. Eosinophils are unstained. They may be difficult to identify and should not be included in the 100 cells selected for scoring.
- 9.4 Other naphthol salts are also available and may give equally good results. Particularly recommended are naphthols AS-CL, AS-TR, AS-AN, or AS-E phosphates. All these diazonium salt fast violet B, and blue dyes when coupled with fast blue RR, BB, or BBN. Fast violet B salt is not the ideal compound to use as the diazonium coupler, but has been found to be the best alternative to fast red violet salt LB, which was originally recommended but is no longer available commercially.

REFERENCES

1. Ackerman, G.A. Substituted naphthol AS phosphate derivatives for the localization of leukocyte alkaline phosphatase activity. *Lab. Invest.* 11: 563-567, 1962.
2. Leonard, B.J., Israëls, M.C.G., and Wilkinson, J.F. Alkaline phosphatase in the white cells in leukaemia and leukaemoid reactions. *Lancet* 1: 289-292, 1958.
3. Kaplow, L.S. Cytochemistry of leukocyte alkaline phosphatase. *Amer. J. Clin. Path.* 39: 439-449, 1963.
4. Kaplow, L.S. Leukocyte alkaline phosphatase cytochemistry: applications and methods. *Ann N.Y. Acad. Sci.* 155: 911-918, 1968.
5. Kaplow, L.S. Leukocyte alkaline phosphatase in disease. *CRC Crit. Rev. Clin. Lab. Sci.*, 1971 pp. 243-278.
6. Okun, D.B. and Tanaka, K.R. Leukocyte alkaline phosphatase. *Amer. J. Hemat.* 4: 293-299, 1978.

RELATED NCCLS PUBLICATIONS

- GP1-A2*** **LABELING OF CLINICAL LABORATORY MATERIALS--SECOND EDITION; APPROVED STANDARD.** Identifies the information necessary for the labeling of materials used in clinical laboratory procedures.
Chairholder: Rosanne M. Savol, Miles Laboratories, Inc.
- H3-A2** **PROCEDURES FOR THE COLLECTION OF DIAGNOSTIC BLOOD SPECIMENS BY VENIPUNCTURE--SECOND EDITION; APPROVED STANDARD.** Procedures for collecting diagnostic blood specimens by venipuncture and a training program for venipuncturists aimed at increasing interlaboratory comparability and minimizing laboratory error.
Chairholder: Jean M. Slockbower, Ph.D., Mayo Clinic
- H4-A** **PROCEDURES FOR THE COLLECTION OF DIAGNOSTIC BLOOD SPECIMENS BY SKIN PUNCTURE; APPROVED STANDARD.** Detailed description and explanation of proper collection and hazards to patients due to inappropriate specimen collection by skin puncture procedures.
Chairholder: Erika Bruck, M.D., American Academy of Pediatrics
- H5-A2** **PROCEDURES FOR THE DOMESTIC HANDLING AND TRANSPORT OF DIAGNOSTIC SPECIMENS AND ETIOLOGIC AGENTS--SECOND EDITION; APPROVED STANDARD.** Proper packaging, handling, and transport requirements for medical specimens including federal regulations that govern these activities.
Chairholder: David J. Sperling, Mayo Clinic
- I1-A2 PREPARATION OF MANUALS FOR INSTALLATION, OPERATION, AND REPAIR OF LABORATORY INSTRUMENTS--SECOND EDITION; APPROVED STANDARD.** Complete description of information needed in a working instrument manual.
Chairholder: David M. Jeffers, York Hospital, Pa.

An (*) following the order code indicates that the document is no longer being reviewed as part of the NCCLS consensus process. However, because of its usefulness to limited segments of the clinical laboratory community, NCCLS is continuing to make this document available for its informational value.