



**SIGMA-ALDRICH®**

**NAPHTHOL AS-D CHLOROACETATE  
ESTERASE AND  
 $\alpha$ -NAPHTHYL ACETATE ESTERASE**  
(Procedure No. 90)

**INTENDED USE**

Sigma-Aldrich reagents are intended for the cytologic demonstration of naphthol AS-D chloroacetate esterase and  $\alpha$ -naphthyl acetate esterase in blood, bone marrow films or tissue touch preparations. Esterase reagents are for "In Vitro Diagnostic Use".

Cellular esterases are ubiquitous and appear to represent a series of different enzymes acting upon select substrates. Under defined reaction conditions, it may be possible to determine hemopoietic cell types, using specific esterase substrates. The described methods provide means to distinguish granulocytes from monocytes.<sup>1,8</sup>

To perform the test, blood, bone marrow films or tissue touch preparations are incubated with either naphthol AS-D chloroacetate or  $\alpha$ -naphthyl acetate in the presence of a stable diazonium salt. Enzymatic hydrolysis of ester linkages liberates free naphthol compounds. These couple with the diazonium salt, forming highly colored deposits at the sites of enzyme activity.

**REAGENTS**

**DIMETHYL FORMAMIDE**, Catalog No. 90-10

**ETHYLENE GLYCOL MONOMETHYL ETHER**,  
Catalog No. 90-11

**NAPHTHOL AS-D CHLOROACETATE**, Catalog No. 90-5  
Capsule contains 20 mg.

**$\alpha$ -NAPHTHYL ACETATE**, Catalog No. 90-6  
Capsule contains 20 mg.

**TRIZMAL™ 6.3 BUFFER CONCENTRATE**,

Catalog No. 90-3C

TRIZMA® maleate, 200 mmol/l. Chloroform added as preservative.

**TRIZMAL™ 7.6 BUFFER CONCENTRATE**,

Catalog No. 90-2C

TRIZMA® maleate, 200 mmol/l. Chloroform added as preservative.

**MAYER'S HEMATOXYLIN SOLUTION**, Catalog No. MHS-1  
Hematoxylin, certified, 0.1% (w/v), and stabilizers.

**ACID HEMATOXYLIN SOLUTION**, Catalog No. 285-2  
Hematoxylin certified, 1 g/l, and stabilizers, pH 3.3 at 25°C.

**FAST BLUE RR SALT**, Catalog No. FBS-25

Preweighed capsules. Actual weight per capsule will vary with dye lot purity and has been optimized by assay.

**FAST CORINTH V SALT**, Catalog No. 90-15

Dye content shown on reagent label.

**CITRATE CONCENTRATE**, Catalog No. 386-1

Citrate buffer, 0.383 mol/l, pH 5.4 when diluted according to procedure.

**GLYCEROL GELATIN**, Catalog No. GG-1

A glycerol-gelatin mixture containing phenol, 1%.

**STORAGE AND STABILITY:**

Dimethyl Formamide, Ethylene Glycol Monomethyl Ether, TRIZMAL™ 6.3 Buffer Concentrate, TRIZMAL™ 7.6 Buffer Concentrate, Mayer's Hematoxylin Solution and Acid Hematoxylin Solution, are stored at room temperature (18–26°C).

Naphthol AS-D Chloroacetate,  $\alpha$ -Naphthyl Acetate and Fast Blue RR Salt are stored below 0°C.

Fast Corinth V Salt and Citrate Concentrate are stored refrigerated (2–8°C).

Naphthol AS-D Chloroacetate,  $\alpha$ -Naphthyl Acetate, Fast Blue RR Salt and Fast Corinth V Salt are stable until the expiration date shown on the labels.

Citrate Dilute Solution is stable for 1 week if stored tightly capped at room temperature (18–26°C).

TRIZMAL™ 6.3 Buffer Concentrate, TRIZMAL™ 7.6 Buffer Concentrate and Citrate Concentrate are suitable for use in the absence of microbial growth.

Sodium fluoride, 2 g/dl. Store at room temperature (18–26°C). Used if " $\alpha$ -Naphthyl Acetate Esterase with Fluoride Inhibition Procedure" is performed.

Glycerol Gelatin, Catalog No. GG-1. Store in refrigerator (2–8°C).

**DETERIORATION:**

Discard Dimethyl Formamide and Ethylene Glycol Monomethyl Ether if colored or turbid.

TRIZMAL™ 6.3 Dilute Buffer Solution and TRIZMAL™ 7.6 Dilute Buffer Solution should be used once then discarded.

Discard Mayer's Hematoxylin and Acid Hematoxylin Solution when the time required for suitable staining exceeds the time recommended in the procedure by more than 5 minutes.

**PREPARATIONS:**

NAPHTHOL AS-D CHLOROACETATE SOLUTION is prepared by dissolving contents of 1 capsule Naphthol AS-D Chloroacetate in 2 ml Dimethyl Formamide, Catalog No. 90-10. Remove 1 capsule from freezer as needed. Prepare immediately prior to use.

$\alpha$ -NAPHTHYL ACETATE SOLUTION is prepared by dissolving contents of 1 capsule  $\alpha$ -Naphthyl Acetate in 2 ml Ethylene Glycol Monomethyl Ether, Catalog No. 90-11. Remove 1 capsule from freezer as needed. Prepare immediately prior to use.

TRIZMAL™ 6.3 DILUTE BUFFER SOLUTION is prepared by diluting 1 part TRIZMAL™ 6.3 Buffer Concentrate with 9 parts deionized water. The pH should be 6.3 at 25°C.

TRIZMAL™ 7.6 DILUTE BUFFER SOLUTION is prepared by diluting 1 part TRIZMAL™ 7.6 Buffer Concentrate with 9 parts deionized water. The pH should be 7.6 at 25°C.

Mayer's Hematoxylin and Acid Hematoxylin Solution should be filtered before use.

CITRATE DILUTE SOLUTION is prepared by diluting 1 part Citrate Concentrate, Catalog No. 386-1, with 9 parts deionized water. pH 5.4 when diluted.

CITRATE-ACETONE-METHANOL FIXATIVE: To 18 ml Citrate Dilute Solution, add 27 ml ACS grade Acetone and 5 ml Methanol, Catalog No. M 1775. Store tightly capped at room temperature (18–26°C). Discard after 8 hours.

**PRECAUTIONS:**

Normal precautions exercised in handling laboratory reagents should be followed. Dispose of waste observing all local, state, provincial or national regulations. Refer to Material Safety Data Sheet for any updated risk, hazard or safety information.

**US Risks and Safety Statements**

Dimethyl Formamide is TOXIC. Harmful by inhalation and in contact with skin. Irritating to eyes and skin. May cause harm to the unborn child. Do not breathe vapor. Wear suitable protective clothing, gloves and eye/face protection. In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible). Combustible - Readily absorbed through skin. Target organs: Liver and kidneys.

Ethylene Glycol Monomethyl Ether is COMBUSTIBLE and TOXIC. Harmful by inhalation, in contact with skin and if swallowed. Irritating to eyes, respiratory system and skin. May impair fertility. May cause harm to the unborn child. In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible). Avoid exposure - obtain special instructions before use. Target organs: Blood and kidneys.

Naphthol AS-D Chloroacetate and  $\alpha$ -Naphthyl Acetate. Caution: Avoid contact and inhalation.

TRIZMAL™ 7.6 Buffer Concentrate and TRIZMAL™ 6.3 Buffer Concentrate are HARMFUL. Harmful if swallowed. Limited evidence of a carcinogenic effect. Harmful: danger of serious damage to health by prolonged exposure through inhalation and if swallowed. Wear suitable protective clothing. Probable carcinogen. Calif. Prop. 65 carcinogen.

Mayer's Hematoxylin Solution is TOXIC. Toxic if swallowed. Irritating to eyes, respiratory system and skin. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Wear suitable protective clothing and gloves. In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible). Target organs: Nerves and liver.

Acid Hematoxylin Solution is TOXIC. Toxic if swallowed. Irritating to eyes, respiratory system and skin. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Wear suitable protective clothing and gloves. In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible). Target organs: Nerves and liver.

Fast Blue RR Salt. Avoid contact and inhalation.

Fast Corinth V Salt is TOXIC. Harmful by inhalation, in contact with skin and if swallowed. May cause cancer. May cause heritable genetic damage. Do not breathe dust. Wear suitable protective clothing, gloves and eye/face protection. In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible).

Citrate Concentrate is CORROSIVE. Causes burns. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Wear suitable protective clothing, gloves and eye/face protection. In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible).

Methanol is FLAMMABLE and TOXIC. Toxic by inhalation, in contact with skin and if swallowed. Toxic: danger of very serious irreversible effects through inhalation, in contact with skin and if swallowed. Irritating to eyes and skin. Keep container tightly closed. Keep away from sources of ignition - no smoking. Take precautionary measures against static discharges. Avoid contact with skin. Wear suitable protective clothing and gloves. In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible).

Acetone is FLAMMABLE and an IRRITANT. Irritating to eyes. Repeated exposure may cause skin dryness or cracking. Vapors may cause drowsiness and dizziness. Keep container in a well-ventilated place. Keep away from sources of ignition - no smoking. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Target organs: Liver and kidneys.

2% Sodium Fluoride Solution. Caution: Avoid contact and inhalation. Target organs: Kidneys and bones.

**EU Risks and Safety Statements**

Dimethyl Formamide is TOXIC. Harmful by inhalation and in contact with skin. Irritating to eyes. May cause harm to the unborn child. In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible). Restricted to professional users. Attention - Avoid exposure - obtain special instructions before use.

Ethylene Glycol Monomethyl Ether is TOXIC. Flammable. Harmful by inhalation, in contact with skin and if swallowed. May impair fertility. May cause harm to the unborn child. In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible). Avoid exposure - obtain special instructions before use.

Naphthol AS-D Chloroacetate and  $\alpha$ -Naphthyl Acetate. Do not breathe dust. Avoid contact with eyes and skin.

Mayer's Hematoxylin Solution is Harmful. Harmful if swallowed.

Acid Hematoxylin Solution is Harmful. Harmful if swallowed.

Fast Blue RR Salt. Do not breathe dust. Avoid contact with eyes and skin.

Fast Corinth V Salt is TOXIC. Harmful by inhalation, in contact with skin and if swallowed. May cause cancer. May cause heritable genetic damage. Do not breathe dust. Wear suitable protective clothing, gloves and eye/face protection. In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible). Avoid exposure - obtain special instructions before use.

Citrate Concentrate is an IRRITANT. Irritating to eyes and skins. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Wear suitable protective clothing and gloves.

Methanol is HIGHLY FLAMMABLE and TOXIC. Highly flammable. Toxic by inhalation, in contact with skin and if swallowed. Toxic: danger of very serious irreversible effects through inhalation, in contact with skin and if swallowed. Keep container tightly closed. Keep away from sources of ignition - no smoking. Wear suitable protective clothing and gloves. In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible).

Acetone is HIGHLY FLAMMABLE and an IRRITANT. Highly flammable. Irritating to eyes. Repeated exposure may cause skin dryness or cracking. Vapors may cause drowsiness and dizziness. Keep container in a well-ventilated place. Keep away from sources of ignition - no smoking. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.

2% Sodium Fluoride Solution. Avoid contact with skin and eyes.

**PROCEDURE**

**SPECIMEN COLLECTION:**

It is recommended that specimen collection be carried out in accordance with NCCLS document M29-A2. No known test method can offer complete assurance that blood samples or tissue will not transmit infection. Therefore, all blood derivatives or tissue specimens should be considered potentially infectious.

Blood, bone marrow films, tissue-touch preparations, and cyto centrifuge preparations may be used with both  $\alpha$ -naphthyl acetate esterase and naphthol AS-D chloroacetate esterase. Either EDTA or heparin will serve as an anticoagulant.<sup>9</sup> Frozen and paraffin embedded tissues may be used with naphthol AS-D chloroacetate esterase.  $\alpha$ -Naphthyl acetate esterase may be used successfully on frozen tissue sections.<sup>10</sup> Blood or bone marrow films may be stored fixed at room temperature (18–26°C) for several weeks or unfixed for several days without appreciable change in activity.<sup>5,9</sup> Do not ship whole blood for assay at other laboratories. Send fixed or unfixed slides. Slides should be kept cool during transit. Allow films to dry at least 1 hour prior to fixation.

**SPECIAL MATERIALS REQUIRED BUT NOT PROVIDED:**

Methanol, Absolute  
 Acetone, ACS Reagent  
 Sodium Fluoride Solution, Catalog No. 91-9  
 Sodium fluoride, 2 g/dl

**NOTES:**

The described procedures are performed at 37°C. If reagents are not at this temperature, weak or negative reactions may be obtained. It is recommended that temperatures be checked with an accurate thermometer. Controlled temperature water baths are more efficient than warm air incubators and should be used for enzyme cytochemical methods. Heat transfer through glass is more rapid than through plastic, thus, glass Coplin jars should be employed.

Many enzyme systems are sensitive to minute traces of detergent. Washing glassware with dilute bleach followed by rinsing in copious quantities of deionized water will prevent detergent effect upon cellular enzymes.

Results are based on a certain degree of subjective interpretation. Individual laboratories should establish their own normal ranges.

The data obtained from this procedure serves only as an aid to diagnosis and should be reviewed in conjunction with other clinical diagnostic tests or information.

**PROCEDURE:****NAPHTHOL AS-D****CHLOROACETATE ESTERASE PROCEDURE:**

1. Fix slides for 1 minute in Citrate-Acetone-Methanol Fixative at room temperature (18–26°C).
2. Wash thoroughly in deionized water and air dry at least 20 minutes.
3. To 50 ml TRIZMAL™ 6.3 Dilute Buffer Solution, PRE-WARMED TO 37°C, add with constant stirring, contents of 1 capsule Fast Corin V Salt, Catalog No. 90-15.
4. When salt is completely dissolved in buffer, add 2 ml Naphthol ASD Chloroacetate Solution. The solution will appear quite turbid.
5. Continue mixing for 15–30 seconds, then add to Coplin jar. DO NOT FILTER.
6. Place specimens in staining solution (from Step 5) and incubate at 37°C for 5 minutes.  
NOTE: PROTECT FROM LIGHT.
7. Remove slides from stain and wash in deionized water for 3 minutes. Discard staining solution.
8. If desired, counterstain in Acid Hematoxylin Solution, Catalog No. 285-2, for 5–10 minutes and wash in tap water.
9. Air dry slides and examine microscopically. If coverslip is required use only an aqueous mounting media.

**α-NAPHTHYL ACETATE ESTERASE PROCEDURE:**

1. Fix slides in Citrate-Acetone-Methanol Fixative for 1 minute at room temperature (18–26°C).
2. Wash thoroughly in deionized water and air dry at least 20 minutes.
3. To 50 ml TRIZMAL™ 7.6 Dilute Buffer Solution, PRE-WARMED TO 37°C, add with constant stirring, contents of 1 capsule Fast Blue RR Salt, Catalog No. FBS-25.
4. When salt is completely dissolved in buffer, add 2 ml α-Naphthyl Acetate Solution. The solution will be yellow and slightly turbid.
5. Continue stirring for 15–20 seconds, then add to Coplin jar. DO NOT FILTER.
6. Place specimens in staining solution (from Step 5) and incubate at 37°C for 30 minutes.  
NOTE: PROTECT FROM LIGHT.
7. Remove slides from stain and wash for 3 minutes in deionized water. Discard staining solution.
8. If desired, counterstain for 5–10 minutes in Mayer's Hematoxylin Solution, Catalog No. MHS-1, and wash in tap water.
9. Air dry slides and examine microscopically. If coverslip is required use only an aqueous mounting media.

**DOUBLE STAINING ESTERASE PROCEDURE**

1. Perform α-Naphthyl Acetate Esterase test as described in Procedure. Do not counterstain.
2. Rinse slides 5 minutes in deionized water.
3. Perform Naphthol AS-D Chloroacetate Esterase test as described in procedure Steps 3-9.

**α-NAPHTHYL ACETATE ESTERASE WITH FLUORIDE INHIBITION PROCEDURE**

Although α-naphthyl acetate esterase is found primarily in cells of monocytic lineage when performed as described, it should be recognized that megakaryocytes and erythroid precursors are positive for this enzyme.<sup>11</sup> Lymphocytes and some mature granulocytes also show occasional positivity.<sup>5</sup> To differentiate these cells conclusively from monocytes, sodium fluoride is incorporated with the incubation system. The monocyte enzyme is inactivated in the presence of this compound.<sup>12</sup> The following procedure may be used to perform the fluoride inhibition test.

1. Fix slides in Citrate-Acetone-Methanol Fixative for 1 minute at room temperature (18–26°C).
2. Wash thoroughly in deionized water and air dry at least 20 minutes.
3. Label 2 beakers A and B, and add the following:

	Beaker A	Beaker B
Prewarmed 37°C TRIZMAL™ 7.6 Dilute Buffer	50 ml	50 ml
Add with constant stirring, Fast Blue RR	1 capsule	1 capsule
α-Naphthyl Acetate Solution	2 ml	2 ml
Sodium Fluoride Solution	—	2 ml

4. Mix well and pour into Coplin jars labeled A and B.
5. Proceed as described in Steps 6–9 of α-Naphthyl Acetate Esterase Procedure.

**PERFORMANCE CHARACTERISTICS****METHOD OF SCORING:**

Scan the film and select a thin area with few erythrocytes. Sites of Naphthol AS-D Chloroacetate Esterase activity will appear as bright red granulation, α-Naphthyl Acetate Esterase as black granulation. Rate from 0 to 4+ on the basis of quantity and intensity of individual dyes within the cytoplasm of the respective cell types. Characteristics of scoring are based somewhat on subjective interpretation. A suggested scoring format is presented in Table 1. Conclusions center on relative presence or absence of staining.

Cell Rating	Intensity of Staining	Interpretation
0	None	–
1+	Faint to Moderate	±
2+	Moderate to Strong	+
3+	Strong	+
4+	Brilliant	+

**RESULTS:****NAPHTHOL AS-D CHLOROACETATE ESTERASE**

This enzyme is usually considered specific for cells of granulocytic lineage. The cells should show red granulation. Activity is weak or absent in monocytes and lymphocytes.

**α-NAPHTHYL ACETATE ESTERASE**

Under the assay conditions (pH 7.6), this enzyme is detected primarily in monocytes, macrophages and histiocytes, and is virtually absent in granulocytes. Monocytes should show black granulation. Lymphocytes may occasionally exhibit activity.

**α-NAPHTHYL ACETATE ESTERASE WITH FLUORIDE INHIBITION**

All cells of monocytic lineage will be negative for enzyme activity, with the exception of differentiated histiocytes or specialized macrophages in tissue which may also be resistant to sodium fluoride.<sup>10</sup>

**DOUBLE STAINING ESTERASE**

Specimens taken through the double staining procedure will demonstrate the granulocytes with red granulation and monocytes with black granulation.

The expected cellular reactivity of tests for esterase activity is summarized in Table II.

Cell Type	Naphthol AS-D Chloroacetate Esterase	α-Naphthyl Acetate Esterase
Myeloblasts	±	±
Promyelocytes	+	±
Neutrophils	+	—
Eosinophils	—	—
Basophils	±	—
Monocytes	—	+
Lymphocytes	—	±
Lymphoblasts	—	±
Megakaryocytes	—	+
Erythroblasts	—	±
Plasma Cells	—	±
Mast Cells	+	—
Hairy Cells	—	±
Histiocytes	±	+

The reagent system should be monitored by the use of positive and negative control slides. Positive control slides may be prepared from leukemic specimens or specific cell lines known to be positive. Cell lines may be obtained from

American Type Culture Collection (ATCC), expanded in culture, frozen and stored in liquid nitrogen. Those suitable for positive controls include: A-937, derived from a human histiocytic lymphoma, for nonspecific esterase; HL-60 a promyelocytic line, for naphthol AS-D chloroacetate esterase; and Molt-4, derived from a T-cell leukemia, for α-naphthyl acetate esterase. Consult the literature accompanying cell lines for proper handling procedures.

Alternately, anti-coagulated blood from normal specimens (preferably with increased monocyte count if using α-naphthyl acetate esterase procedure) may also be used; however, they will provide less intense staining and will have fewer positive cells.

Known negative patient slides may be used as a negative control. If unavailable, staining a specimen in an incubation mixture with the substrate omitted will give the desired results. However, use of the former is highly recommended.

If observed results vary from expected results, please contact Sigma-Aldrich Technical Service for assistance.

**REFERENCES**

1. Beard, MEJ, Fairly GH: Acute leukemia in adults. *Semin Hematol* 11:5, 1974.
2. Beckmann H, Neth R, Gaedicke G, Lanbeck G, Schoch G, Wieggers U, Winkler K: Cytology and cytochemistry of the leukemic cell. *Haematol Bluttransfus* 14:26, 1974.
3. Bennet JM, Reed CE: Acute leukemia cytochemical profile: Diagnostic and clinical implications. *Blood Cells* 1:101, 1975.
4. Cawley JC, Hayhoe FGJ: Acute leukemia: Cellular morphology, cytochemistry and fine structure. *Clinics in Haematol* 1:49, 1972.
5. Yam LT, Li CY, Crosby WH: Cytochemical identification of monocytes and granulocytes. *Am J Clin Pathol* 55:283, 1971.
6. Yam, LT, Li CY, Wolfe HJ, Moy PW: Histochemical study of acute leukemia. *Arch Pathol* 97:129, 1974.
7. Burstone MS: The cytochemical localization of esterase. *J Natl Cancer Inst* 18:167, 1957.
8. Moloney WC, McPherson K, Slierman L: Esterase activity in leukocytes demonstrated by the use of naphthol AS-D chloroacetate substrate. *J Histochem Cytochem* 8:200, 1960.
9. Brown BA: *IN Hematology: Principles and Procedures*. Lea and Febiger, Philadelphia, PA, 1984, pp 127–130.
10. Sun T: *Atlas of Cytochemistry and Immunocytochemistry of Hematologic Neoplasms*. American Society of Clinical Pathologists Press, Chicago, IL, 1985, pp 24, 38.
11. Hayhoe FGJ, Flemans RJ: *IN Color Atlas of Hematological Cytology*. John Wiley & Sons, New York, NY, 1982, pp 34, 111.
12. Li CY, Lam KW, Lam LT: Esterases in human leukocytes. *J Histochem Cytochem* 21:1, 1973.

Sigma-Aldrich, Inc. warrants that its products conform to the information contained in this and other Sigma-Aldrich publications. Purchaser must determine the suitability of the product(s) for their particular use. Additional terms and conditions may apply. Please see reverse side of the invoice or packing slip for additional terms and conditions of sale.

Procedure No. 90  
 Previous Revision: 2003-09  
 Revised: 2006-04



AR-MED Ltd., Runnymede Malthouse  
 Egham TW20 9BD United Kingdom

SIGMA-ALDRICH, INC.  
 3050 Spruce Street, St. Louis, MO 63103 USA  
 314-771-5765  
 Technical Service: 800-325-0250  
 or call collect 314-771-3122  
 or e-mail at [clintech@sial.com](mailto:clintech@sial.com)  
 To Order: 800-325-3010  
 or call collect 314-771-5750  
[www.sigma-aldrich.com](http://www.sigma-aldrich.com)

SIGMA-ALDRICH CHEMIE GmbH  
 P.O. 1120, 89552 Steinheim, Germany  
 49-7329-970