SIGMA-ALDRICH®

NAPHTHOL AS-D CHLOROACETATE ESTERASE AND α-NAPHTHYL ACETATE ESTERASE

(Procedure No. 91)

INTENDED USE

For the cytologic demonstration of specific and non-specific leukocyte esterase. Esterase reagents are for "In Vitro Diagnostic Use".

Cellular esterases are ubiquitous, apparently representing 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 hematologists and hematopathologists means of distinguishing granulocytes from monocytes.[1-8]

To perform the test, blood, bone marrow films or tissue touch preparations are incubated with either naphthol AS-D chloroacetate (NCAE) or α-naphthyl acetate (NAE) in the presence of freshly formed diazonium salt. Enzymatic hydrolysis of ester linkages liberates free naphthol compounds. These couple with the diazonium salt, forming highly colored deposits at sites of enzyme activity.

Most recent procedures, including those provided by Sigma-Aldrich, employ stable diazonium salts. These are formed by reacting an arylamine with sodium nitrite in an acid medium.[9] The resulting diazonium chloride (usually unstable) can then be treated with compounds such as zinc chloride, zinc sulfate or naphthalene-1-6-disulfonate, forming stable salts. These stabilizers may exert marked inhibition on some enzymatic systems, whereas the diazonium chlorides are less inhibitory.[9] For this reason, Sigma-Aldrich now provides stable solutions for Fast Red Violet LB Base, Fast Blue BB Base and Sodium Nitrite for esterase cytochemistry. To further simplify these methods, stable solutions of Naphthol AS-D Chloroacetate and α-Naphthyl Acetate are included. The availability of these stable solutions allows the customer to adjust working reagent volumes according to needs, eliminating waste.

REAGENTS

NAPHTHOL AS-D CHLOROACETATE SOLUTION, Catalog No. 911-10 ml
Naphthol AS-D chloroacetate, 8 mg/ml, and stabilizer.

FAST RED VIOLET LB BASE SOLUTION, Catalog No. 912-10 ml
Fast red violet LB base, 15 mg/ml, in 0.4 mol/l hydrochloric acid with stabilizer.

TRIZMAL™ 6.3 CONCENTRATE, Catalog No. 913-50 ml
TRIZMA® maleate, 1 mol/l, with surfactant. pH 6.3 ± 0.15 at 25°C.

SODIUM NITRITE SOLUTION, Catalog No. 914-10 ml
Sodium nitrite, 0.1 mol/l.

CITRATE SOLUTION, Catalog No. 915-50 ml
Citric acid, 18 mmol/l, sodium citrate, 9 mmol/l, sodium chloride, 12 mmol/l, with surfactant. pH 3.6 ± 0.1 at 25°C.

α-NAPHTHYL ACETATE SOLUTION, Catalog No. 916-10 ml
α-Naphthyl acetate, 12.5 mg/ml, in methanol solution with stabilizers.

FAST BLUE BB BASE SOLUTION, Catalog No. 917-10 ml
Fast blue BB base, 15 mg/ml in 0.4 mol/l hydrochloric acid with stabilizers.

TRIZMAL™ 7.6 CONCENTRATE, Catalog No. 918-50 ml
TRIZMA® maleate, 1 mol/l, with surfactant. pH 7.6 ± 0.15 at 25°C.

HEMATOXYLIN SOLUTION GILL NO. 3, Catalog No. GHS3-50 ml
Certified hematoxylin, 6.0 g/l, sodium iodate, 0.6 g/l, and aluminum sulfate, 52.8 g/l, with stabilizers.

SODIUM FLUORIDE SOLUTION, Catalog No. 919-25 ml
Sodium Fluoride, 20 g/l

STORAGE AND STABILITY:

Store Hematoxylin Solution Gill No. 3 at room temperature (18–26°C) protected from light. Store other reagents in refrigerator (2–8°C).

TRIZMAL™ 6.3 Concentrate, TRIZMAL™ 7.6 Concentrate and Citrate Solution are suitable for use in the absence of microbial growth. Other reagents are stable until expiration date shown on the labels.

DETERIORATION:

Discard TRIZMAL™ Concentrate and Citrate Solution if microbial growth is evident. Discard Hematoxylin Solution Gill No. 3 if solution turns brown (over-oxidized from air) or purple (loss of acidity).

PREPARATION:

Warm all reagents to room temperature (18–26°C) before use. Esterase reagents are provided ready for use.

Citrate-Acetone-Formaldehyde Fixative: To 25 ml Citrate Solution, add 65 ml Acetone and 8 ml 37% Formaldehyde. Place in glass bottle and cap tightly. Store in refrigerator (2–8°C). Bring to room temperature (18–26°C) prior to use. Stable up to 4 weeks if stored tightly capped in refrigerator

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 and product labeling for any updated risk, hazard or safety information.

PROCEDURES

SPECIMEN COLLECTION:

It is recommended that specimen collection be carried out in accordance with CLSI document M29-A3. 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 preparation and cytocentrifuge preparations may be used with both α-naphthyl acetate esterase and naphthol AS-D chloroacetate esterase. Either EDTA or heparin will serve as an anticoagulant.[10] Frozen and paraffin embedded tissues may be used with naphthol AS-D chloroacetate esterase. α-Naphthyl acetate esterase may be used successfully on frozen tissue sections.[11] 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.[5,10] 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 MATERIAL REQUIRED, BUT NOT PROVIDED:

Acetone, ACS Reagent
Formaldehyde, 37%, ACS
Sodium Fluoride Solution, Catalog No. 919-25 ml (Required for α-Naphthyl Acetate Esterase with Fluoride Inhibition Procedure.)

LIMITATIONS OF THE PROCEDURE:

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.

The fixative system described in the "Reagents" section contains formaldehyde. If fixative is not completely removed by vigorous rinsing, any amount of aldehyde, either concentrated on air-dried slides or trace amounts from wet slides, added to the incubation system may result in enzyme inhibition. To prevent loss of blood film during the rinse process, direct the water stream onto the slide above the feather edge. Rinse both sides of the slide.

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.

NOTE:

Users of Sigma Kits 390-A and 91-C should exercise caution when using TRIZMA® Buffer Concentrates Catalog Nos. 91-3 and 90-3C as they are not interchangeable. Use of incorrect buffer will result in a negative reaction.

PROCEDURE:

The described procedures are performed at 37°C

NAPHTHOL AS-D CHLOROACETATE ESTERASE PROCEDURE

1. Prewarm sufficient deionized water for substrate use to 37°C. Check temperature before use.
2. Immediately prior to fixation, add 1 ml Sodium Nitrite Solution to 1 ml Fast Red Violet LB Base Solution in a test tube. Mix gently by inversion and allow to stand 2 minutes. Active evolution of gas bubbles should be avoided.
3. Add solution from Step 2 to 40 ml prewarmed deionized water from step 1.
4. Add 5 ml TRIZMAL™ 6.3 Buffer Concentrate. (See "Note")
5. Add 1 ml Naphthol AS-D Chloroacetate Solution. The solution should turn red. Mix well and pour into Coplin jar.
6. Bring Citrate-Acetone-Formaldehyde (CAF) Solution to room temperature (18–26°C). Fix slides by immersing in CAF solution for 30 seconds.
7. Rinse slides thoroughly in running deionized water for 45–60 seconds, then place in solution from Step 5. Do not allow slides to dry.
8. Incubate for 15 minutes, at 37°C protected from light.
9. After 15 minutes, remove slides and rinse thoroughly in deionized water for at least 2 minutes.
10. Counterstain 2 minutes in Hematoxylin Solution, Gill No. 3.
11. Rinse in tap water and air dry.
12. Evaluate microscopically. If coverslipping is required use only an aqueous mounting media.

NOTES:

1. For use with Columbia jars, divide reagent volumes by 5.
2. If substrate (See Step 5) appears turbid bring to room temperature (18–26°C) and mix well.
3. If slides have been prefixed and stored, skip fixation (steps 6 and 7) and begin staining of dry, prefixed slides at Step 8.

α-NAPHTHYL ACETATE ESTERASE PROCEDURE

1. Prewarm sufficient deionized water for substrate use to 37°C. Check temperature before use.
2. Immediately prior to fixation, add 1 ml Sodium Nitrite Solution to 1 ml Fast Blue BB Base Solution in a test tube. Mix by inversion and allow to stand at least 2 minutes. The color will change from dirty brown to deep yellow. Active evolution of gas bubbles should be avoided.
3. Add solution from Step 2 to 40 ml prewarmed deionized water from step 1.
4. Add 5 ml TRIZMAL™ 7.6 Buffer Concentrate.
5. Add 1 ml α-Naphthyl Acetate Solution. The solution should turn greenish. Mix well and pour into Coplin jar.
6. Bring Citrate-Acetone-Formaldehyde (CAF) Solution to room temperature (18–26°C). Fix slides by immersing in CAF solution for 30 seconds. Agitate slides vigorously for the last 5 seconds.
7. Rinse slides thoroughly in running deionized water for 45–60 seconds, then place in solution from Step 5. Do not allow slides to dry.
8. Incubate for 30 minutes at 37°C protected from light.
9. After 30 minutes, remove slides and rinse thoroughly for at least 2 minutes in running deionized water.
10. Counterstain 2 minutes in Hematoxylin Solution, Gill No. 3.
11. Rinse in tap water and air dry.
12. Evaluate microscopically. If coverslipping is required use only an aqueous mounting media.

NOTES:

1. For use with Columbia jars, divide reagent volumes by 5.
2. If substrate (See Step 5) appears turbid bring to room temperature (18–26°C) and mix well.
3. If slides have been prefixed and stored, skip fixation (steps 6 and 7) and begin staining of dry, prefixed slides at Step 8.

DOUBLE STAINING ESTERASE PROCEDURE

1. Perform α-Naphthyl Acetate Esterase test as described in Procedure. Do not counterstain.
2. Rinse slide 5 minutes in deionized water.
3. Perform Naphthol AS-D Chloroacetate Esterase test as described in Procedure Steps 1-12. Omit Step 6.

NOTE:

Fast Blue BB Base Solution may be substituted for Fast Red Violet LB Base Solution if blue granulation is preferred for Naphthol AS-D Chloroacetate Esterase.

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.[12] Lymphocytes and some mature granulocytes also show occasional positivity.[5] 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.[13] The following procedure may be used to perform the fluoride inhibition test.

1. To 2 ml Fast Blue BB Base Solution add 2 ml Sodium Nitrite Solution. Mix gently by inversion. Allow to stand 2 minutes.
2. Label 2 beakers A and B, and add the following:

	Beaker A	Beaker B
Prewarmed 37°C deionized water	40 ml	40 ml
Diazotized Fast Blue BB from Step 1	2 ml	2 ml
TRIZMAL™ 7.6 Concentrate	5 ml	5 ml
α-Naphthyl Acetate Solution	1 ml	1 ml
Sodium Fluoride Solution	—	1 ml

3. Mix well and pour into Coplin jars labeled A and B.
4. Proceed as described in Steps 6-12 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 on somewhat subjective interpretation. A suggested scoring format is presented in Table 1. Conclusions center on relative presence or absence of staining.

TABLE I
Characteristics of Scoring

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

EXPECTED OBSERVATIONS:

NAPHTHOL AS-D CHLOROACETATE ESTERASE

(Fast Red Violet LB) - Enzyme is usually considered specific for cells of granulocytic lineage. Sites of activity show bright red granulation. Activity is weak or absent in monocytes and lymphocytes.

α-NAPHTHYL ACETATE ESTERASE:

(Fast Blue BB) - Enzyme is detected primarily in monocytes, macrophages and histiocytes, and is virtually absent in granulocytes. Monocytes should show black granulation. Lymphocytes may occasionally exhibit enzyme 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.[11]

DOUBLE STAINING ESTERASE

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

QUALITY CONTROL:

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.

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.

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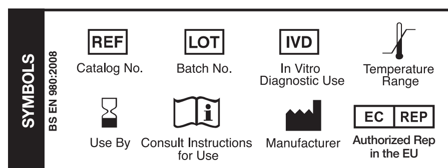

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