SIGMA-ALDRICH®

ALKALINE PHOSPHATASE

(Procedure No. 85)

INTENDED USE

Sigma-Aldrich Alkaline Phosphatase kits are intended for the histochemical semi-quantitative demonstration of alkaline phosphatase activity in leukocytes. Alkaline Phosphatase reagents are for "In Vitro Diagnostic Use".

In 1929, Kay first suggested the presence of alkaline phosphatase in leukocytes.[1] However, not until many years later did Kaplow[2] introduce a practical staining method for demonstrating the leukocyte enzyme. Kaplow[2] used sodium α-naphthyl phosphate as substrate, fast blue RR as the diazonium salt and recommended a scoring method for semiquantitative comparisons. Also suggested were improvements resulting in good preservation of cellular morphology. Subsequently, α-naphthyl phosphate was replaced by naphthol AS phosphate. It is noteworthy that a good correlation has been found between cytochemical and biochemical techniques for assaying leukocyte alkaline phosphate activity (LAPA).[3]

With the Sigma-Aldrich method, which is essentially that of Ackerman,[4] leukocyte alkaline phosphatase is determined in blood or bone marrow films after they are gently fixed to slides. The fixed films are then incubated in a solution containing naphthol AS-MX phosphate. As a result of phosphatase activity, naphthol AS-MX is liberated and immediately coupled with a diazonium salt, forming an insoluble, visible pigment at sites of phosphatase activity.

REAGENTS

FAST BLUE RR SALT, Catalog No. FBS25-10 CAP
Preweighed capsules. Actual weight per capsule will vary with dye lot and has been optimized by assay.

FAST VIOLET B SALT, Catalog No. 851-10 CAP
Capsule contains 12 mg.

NAPHTHOL AS-MX PHOSPHATE ALKALINE SOLUTION, Catalog No. 855-20 ml
Naphthol AS-MX phosphate, 0.25% (w/v), buffered at pH 8.6, 25°C

MAYER'S HEMATOXYLIN SOLUTION, Catalog No. MHS1-100 ml
Hematoxylin, certified, 1 g/l, sodium iodate, 0.2 g/l, aluminum ammonium hydroxide, 5 g/l, and stabilizers.

CITRATE CONCENTRATED SOLUTION, Catalog No. 854C-20 ml
Contains citric acid-sodium citrate, 1.5 mol/l.

STORAGE AND STABILITY:

Store Fast Blue RR Salt and Fast Violet B Salt below 0°C. Store Naphthol AS-MX Phosphate Alkaline Solution in refrigerator (2–8°C). Reagents are stable until expiration date.

Store Mayer's Hematoxylin Solution tightly capped at room temperature (18–26°C) protected from light. Do not return solution to original container after use in Coplin jar. Discard when the time required for suitable staining exceeds the time recommended in the procedure by more than 5 minutes.

Store Citrate Concentrated Solution at room temperature (18–26°C). Store Citrate Working Solution in refrigerator (2–8°C). Citrate solutions are suitable for use in the absence of microbial growth.

PREPARATION:

Fast Blue RR Salt, Fast Violet B Salt, Naphthol AS-MX Phosphate Alkaline Solution and Mayer's Hematoxylin Solution are provided ready for use in the procedure.

Prepare Citrate Working Solution by diluting 2 ml Citrate Concentrate Solution to 100 ml with deionized water.

To prepare Fixative Solution (citrate buffered acetone, 60%), warm Citrate Working Solution to room temperature (18–26°C). With constant stirring, add 2 volumes of Citrate Working Solution to 3 volumes of acetone. Discard after use.

Scott's Tap Water Substitute Working solution is prepared by diluting 1 volume of Scott's Tap Water Substitute Concentrate with 9 volumes deionized water.

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.

PROCEDURE

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.

Fresh blood or bone marrow films or samples anticoagulated with heparin may be used.[5] AVOID EDTA.[5] Blood smears should be stained for enzyme activity within 8 hours after preparation. However, if this is not possible, gradual loss of alkaline phosphatase activity may be delayed by fixation and storage overnight in freezer.[5] Films should be dried at least 1 hour prior to fixation and 3 hours post-fixation before freezing.

SPECIAL MATERIALS REQUIRED BUT NOT PROVIDED:
Scott's tap water substitute concentrate, Catalog No. S5134
Acetone, ACS reagent.

NOTES:

There is a scarcity of data concerning compounds which may interfere with leukocyte alkaline phosphatase activity (LAPA). Certain drugs and other substances are known to influence circulating alkaline phosphatase activity.[6] Oral contraceptives, cortisol and stress may result in elevated leukocyte alkaline phosphatase scores.[7]

Perform procedure using positive controls. These can be obtained from patients with pyogenic leukocytosis, or women in the third trimester of pregnancy or during the first several days postpartum. Leukocyte alkaline phosphatase scores from these persons usually exceed 100. A negative control can be prepared from a normal fixed smear by immersing it in boiling water for 1 minute to inactivate the enzyme. Control films can be preserved up to 1 year if stored fixed, wrapped in Parafilm® at –70°C. These films should be air dried at least 1 hour prior to fixation and 3 hours post-fixation before freezing.

It is strongly recommended that each laboratory establish it's own expected range, characteristic for the local population.

The procedure depends upon subjective rating of stained cells. This can result in a wide variation of ratings obtained. The temperature of the reaction mixture must be kept between 18–26°C. Lower temperatures will result in significantly lower scores. Above 30°C, marked increases in activity will occur. Eosinophils do not stain but can be recognized by bilobate nuclei and large refractile granules.

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:

1. Measure 48 ml distilled water into a suitable container and adjust temperature to 18–26°C
2. Prepare diazonium salt solution:
   Dissolve contents of one Fast Blue RR Salt capsule or one Fast Violet B capsule in distilled water (from step 1). A magnetic stirrer may be helpful.
3. Add 2 ml Naphthol AS-MX Phosphate Alkaline Solution to diluted diazonium salt solution (from step 2). Mix.
4. Bring Fixative Solution to room temperature (18–26°C). Fix slides by immersing in Fixative Solution for 30 seconds. Rinse gently in deionized water for 45seconds. Do not allow slides to dry.
5. Add slides to alkaline-dye mixture (from step 3) and incubate at 18–26°C for 30 minutes. Protect immersed slides from direct light. Discard alkaline-dye mixture after use.
6. After 30 minutes, remove slides and rinse thoroughly in deionized water for 2 minutes. Do not allow slides to dry.
7. Place slides in Mayer's Hematoxylin Solution for 10 minutes.
   NOTE: If using Fast Blue RR Salt, rinse counterstained slides for 3 minutes in deionized water. This will result in red violet nuclear staining. If using Fast Violet B Salt, rinse counterstained slides in tap water (if alkaline) or immerse in Scott's Tap Water Substitute for 2 minutes. This will result in blue nuclear stain.
8. Evaluate microscopically. If coverslipping is required use only an aqueous mounting media.

PERFORMANCE CHARACTERISTICS

METHOD OF SCORING:

Scan the film (900X) and select a thin area where erythrocytes are barely touching. Sites of phosphatase activity will appear as blue or red granules, depending upon dye used. Select 100 consecutive segmented and band form neutrophilic granulocytes. Rate from 0 to 4 + on the basis of quantity and intensity of precipitated dye within the cytoplasm of these cells. For characteristics of scoring, refer to Table 1. The sum of the ratings of 100 cells is regarded as the score.

TABLE 1. CHARACTERISTICS OF SCORING*

	Precipitated Azo Dye in Cytoplasm			
Cell Rating	Amount** (%)	Size of Granule	Intensity of Staining	Background of Cytoplasma
0+	None	—	None	None
1+	50	Small	Faint to Moderate	Colorless to very pale pink or blue
2+	50–80	Small	Moderate to Strong	Colorless to pale pink or blue
3+	80–100	Medium to Large	Strong	Colorless to pink or blue
4+	100	Medium and Large	Brilliant	Not visible

* Table 1 represents modification of observations made by Kaplow.[2,3]

** Percentage of volume of cytoplasm occupied by azo dye precipitate.

To obtain the leukocyte alkaline phosphatase activity (LAPA) score, the number of cells counted is multiplied by the value for cell rating. These figures are added to obtain the LAPA score as shown in the following examples:

Cell Rating	Number Counted	LAPA Score
0	60	0
1+	20	20
2+	14	28
3+	5	15
4+	1	4
Total	100	67

EXPECTED VALUES:

The following scores were obtained at Sigma-Aldrich for 20 normal individuals. Blood was drawn into heparinized tubes and films were prepared within one hour.

	Fast Blue RR	Fast Violet B
LAPA Mean ± 1 SD	96±44	72±50
LAPA Range	52–140	22–122

The range of normal scores is wide, varying from 12 to 182. The following range of scores were obtained from those 20 normal individuals:

Fast Blue RR	Fast Violet B
32–182	12–180

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

EXPECTED OBSERVATIONS:

In humans, alkaline phosphatase activity is restricted to mature and band-form granulocytes. Occasionally weak staining may be observed in lymphocytes. Bone marrow osteoblasts and endothelial cells stain strongly. Marked increase in peripheral blood leukocyte alkaline phosphatase is observed in multiple myeloma, Hodgkin's disease, polycythemia vera and infectious leukocytosis. Low or absence of activity is seen in chronic myelocytic leukemia, hereditary hypophosphatasia and paroxysmal noncturnal hemoglobinuria.

A series of slides was prepared using blood obtained from normal subjects, females in the last trimester of pregnancy, early postpartum patients, and individuals exhibiting a leukemoid reaction. Negative controls were prepared by heat inactivation as described. Various combinations (Fast Blue RR and Fast Violet B) and counterstains (Methylene Blue Solution and Mayer's Hematoxylin Solution) were performed in replicate and the LAPA scored. Values obtained on multiple slides prepared on each subject were in close agreement and did not appear influenced by the staining/counterstaining technique employed. For example, 7 smears made using blood from a prenatal patient yielded scores ranging from 235–269 with a mean, standard deviation and coefficient of variation of 255, 11.3 and 4.4% respectively. Negative controls revealed a complete absence of stained material.

REFERENCES

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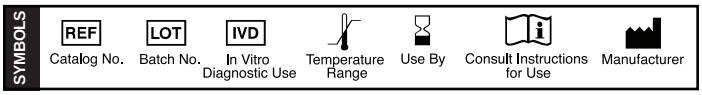

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