

INTENDED USE

For the histochemical demonstration of intracytoplasmic NBT reduction in neutrophils to identify neutrophil dysfunction and/or distinguish pyrogenic infection.² NBT staining reagents are for "In Vitro Diagnostic Use."

Park and his associates¹ are credited with being first to apply the nitroblue tetrazolium (NBT) neutrophil reduction test as a diagnostic aid in differentiating bacteria-induced febrile conditions from those nonbacterial in origin. The test involved incubation of blood with a buffered solution of NBT. Smears are prepared, stained and examined microscopically to determine the percentage of neutrophils showing intracytoplasmic deposits of formazan. This percentage is usually increased in bacterial infections.

Some disease states, especially those involving metabolic defects of neutrophil function, show low or normal NBT test values even when active bacterial infection is present. These conditions may be detected by modification of the NBT test to include *in vitro* stimulation of the phagocytic system. This stimulation may be accomplished by incorporation of bacterial culture filtrate,^{2,3} latex particles,^{4,5} zymosan,⁶ endotoxin,⁷⁻⁹ glass contact,⁶ or high concentrations of heparin,⁹⁻¹² into the blood-NBT incubation mixture. *In vitro* stimulation of blood from normal persons, without cellular or humoral defects and without impairment in granulocyte metabolism, will show marked increase in the percent of formazan containing neutrophils. Cells of patients with such defects (e.g., chronic granulomatous disease, CGD) fail to exhibit a positive response, even when stimulated.^{4,6,8,11}

Numerous papers have appeared^{4,7,13-27} either confirming or denying the original claims¹ concerning the diagnostic usefulness of this test. In a review of the current status of the NBT test in clinical diagnosis, Segal²⁸ suggests it is of little value in the diagnosis of pyrogenic infection. In reply to this criticism, Freeman and King²⁹ point out that conflicting results from different laboratories may arise from use of poorly standardized modifications of the original Park procedure.¹

Technical factors reported to affect results in the NBT test are:

1. Duration and temperature at which blood is stored before assay.^{9,10}
2. Duration and temperature at which blood-NBT mixture is incubated.^{6,9-12,30-34}
3. Concentration of heparin or NBT.^{5,9-12,31-34}
4. Capillary blood versus venous blood.³⁵
5. Plastic versus siliconized glass contact during incubation.³⁴
6. Observer experience.^{15,29}
7. Siliconized versus nonsiliconized glass contact during storage or incubation.⁵
8. Collection in Vacutainer[®] tubes.²⁴
9. Criteria used for identifying cells as positive or negative.^{2,11,26,33,35}
10. Use of EDTA as anticoagulant; resulting inhibition of NBT response.^{3,12}

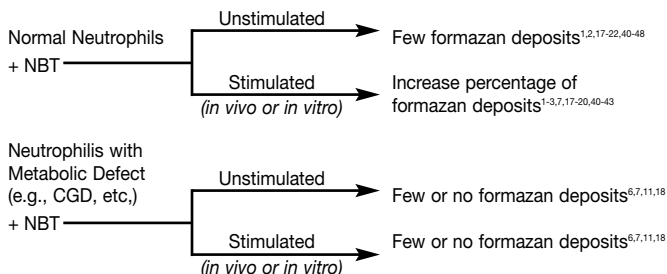
The inhibitory effect of EDTA is apparently abolished if the test is carried out on buffy coats prepared from whole blood in the presence of Ficoll[®], a sucrose polymer.³³ Ficoll[®] is said to exert a protective effect on the cytoplasmic membrane of leukocytes during their incubation with NBT.³⁶ The use of buffy coats to concentrate neutrophils, thereby shortening enumeration time, has also been suggested by Patterson³⁷ and others.^{20,38}

Because of the need for standardization, Sigma-Aldrich offers a semiquantitative NBT procedure, based on a modification of the method of Feigin et al.^{17,34} that is derived from the reference method of Park and associates.¹

The NBT test has been proposed as an aid in:

1. Identifying patients with chronic granulomatous disease or similar conditions due to metabolic defects of neutrophil function.^{4-6,11,39}
2. Distinguishing febrile conditions and/or leukocytoses of bacterial infection from those of nonbacterial origin.^{1,7,17,18}
3. Determining response to antibiotic therapy.^{1,2,7,17-19}
4. Monitoring patients with high susceptibility to bacterial infection.^{7,20}

To perform the test, heparinized blood samples are incubated with a buffered solution of NBT under carefully controlled conditions.^{1,11,34} Smears are then prepared, stained and examined microscopically to determine the percentage of neutrophils showing intracytoplasmic deposits of reduced NBT (formazan).



The performance of a "stimulated" procedure may prove useful in revealing the presence of an intrinsic neutrophil defect.^{4,6,7,11,18,42,49,50}

REAGENTS

NBT VIAL, Catalog No. 840-10

Nitroblue tetrazolium, 1 mg, lyophilized, with phosphate buffer and sodium chloride.

HEPARIN(N), SODIUM SALT, Catalog No. 840-20

Silanized glass vials containing heparin(N) (porcine), 20 units, for collection of 1-ml whole blood.

VIALS, GLASS WITH CAPS, Catalog No. 840-50

Siliconized vials for incubation of samples.

ACCUSTAIN[®] WRIGHT STAIN, Catalog No. WS 10

Wright stain, 0.3%, buffered at pH 6.8, in methanol.

STIMULANT, Catalog No. 840-15

Bacterial extracts (nonviable), lyophilized.

STORAGE AND STABILITY:

Store NBT vial in the dark, refrigerated (2–8°C). Store Stimulant refrigerated (2–8°C). Store Heparin and vials at room temperature (18–26°C).

Store ACCUSTAIN Wright Stain at room temperature (18–26°C). Stable until the expiration date shown on the label.

PREPARATION:

NBT SOLUTION is prepared by reconstituting NBT Vial, Catalog No. 840-10, with 1.0 ml distilled water. Let stand a few minutes then mix vigorously. Reconstituted vial is stable for 1 day stored in refrigerator (2–8°C).

STIMULANT SOLUTION is prepared by reconstituting Stimulant, Catalog No. 840-15, with 1.5 ml distilled water. Shake to dissolve. Store below 0°C. Solution may be frozen and thawed several times.

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 Risk and Safety Statements

NBT Vials. Caution: Avoid contact and inhalation.

Heparin Vials. Caution: Avoid contact and inhalation. Target organ: Blood

Stimulant. Caution: Substance not yet fully tested

Wright Stain Solution is FLAMMABLE and TOXIC. Toxic by inhalation, in contact with skin and if swallowed. Irritating to eyes and skin. Keep away from sources of ignition – no smoking. Keep container tightly closed. Wear suitable protective clothing and gloves. In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible).

EU Risk and Safety Statements (Caution: Substances not yet fully tested)

NBT Vials. Avoid contact with skin and eyes. Do not breathe dust.

Stimulant. Caution: Substance not yet fully tested

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

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 should not be collected more than 2 hours before performing the test. If not immediately tested, sample should be stored in refrigerator.^{9,33} A plastic syringe is used for venipuncture. Introduction of tissue juices should be avoided. The needle is removed from syringe before gently expelling exactly 1 ml blood into a siliconized collection vial containing 20 units of Heparin, Catalog No. 840-20. Mix gently, but well, by tilting slightly and "rolling" the vial for approximately 30 seconds. Avoid contact of blood with cap.

SPECIAL MATERIALS REQUIRED BUT NOT PROVIDED:

Microscope with oil immersion objective.

Pipeting devices for the accurate delivery of volumes required for the assay

Water bath, 37°C

Microscope slides

NOTES:

When the procedure is performed employing the reagent with a normal blood sample, an elevated response should be obtained. If this does not occur, the reagent may have deteriorated.

A thick smear provides a greater number of neutrophils, particularly important when relative and absolute numbers of neutrophils are low, thereby allowing faster numeration.

Each laboratory should establish its own optimum staining time.

It is recommended that each laboratory establish a normal range. Blood from normal individuals should be subjected to both described procedures as controls with each series of tests performed. If reagent system is functioning satisfactorily, the number of formazan-containing cells will be increased above normal after stimulation of control with the bacterial extract.

The quantitative response of clinically healthy persons obtained with the stimulated NBT test varies considerably,^{2,3} making interpretation difficult. However, NBT test values will usually be increased by an additional 10–50% positive cells due to the presence of various stimulants.^{2,3,9,24} For example, an unstimulated sample which yielded 10% positive cells might be expected after stimulation to yield 20 to 60% positive cells. The use of Stimulant, Catalog No. 840-15, as described above, should yield an elevated response in blood from healthy individuals.

There have been reports of elevated values when whole blood is replaced in the reaction mixture by cerebrospinal fluid from cases of bacterial meningitis,⁴⁴ or synovial fluid from cases of pyrogenic arthritis.⁵³ Use of body fluids other than whole blood has not been fully evaluated and judgements relative to their use with reagents provided cannot be offered at this time.

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:

Unstimulated:

A stimulated NBT test (treatment of blood with bacterial extract) may be performed as a positive control in conjunction with, or subsequent to, this unstimulated NBT test in order to detect metabolic defects of neutrophil function. The stimulated NBT test is also described.

Incubation of Sample and Preparation of Smears

1. With a plastic pipet, transfer 0.12 ml NBT Solution to a vial, Catalog No. 840-50.
2. Add 0.2 ml well-mixed heparinized blood. Mix gently, but well, by tilting slightly and “rolling” the vial. Do not invert the vial. Cap tightly.
3. Incubate at 37°C for 10 minutes. Remove and let stand at room temperature (18–26°C) an additional 10 minutes.
4. Mix heparinized blood-NBT mixture again by “rolling” gently.
5. With a plastic pipet, transfer 50–75 µL of mixture onto a clean glass slide.
NOTE: Care should be taken to minimize mechanical damage to white blood cells during smear preparation.
6. Prepare smear moderately thick in order to reduce mechanical damage to formazan-containing cells, which become more fragile.^{6,34} Permit smear to air dry.
7. Treat smear with ACCUSTAIN Wright Stain, Catalog No. WS 10, as follows:
 - a. Flood dried smear with 1 ml of stain for 15 seconds.
 - b. To flooded smear add 1 ml of distilled water and allow to stand for 30 seconds (longer times increase stain intensity).
 - c. Rinse smear with water, allow to drain and blot or air dry.

Stimulated:

This procedure may be performed simultaneously with, or subsequent to, the unstimulated NBT test as a possible aid in detecting metabolic defects of neutrophil function (refer to “Intended Use” section).

Incubation of Sample and Preparation of Smears:

1. Transfer 0.1 ml NBT Solution to a vial, Catalog No. 840-50.
2. With a plastic pipet, add 0.05 ml heparinized blood and 5 µL Stimulant Solution. Mix gently, but well, by tilting slightly and “rolling” the vial. Cap tightly.
3. Proceed with Steps 3 through 7 as in “Procedure (Unstimulated)” and continue with “Microscopic Examination and Counting” section.

PERFORMANCE CHARACTERISTICS

Test values are reported in terms of percent positive (formazan-containing) neutrophils.

Microscopic Examination and Counting:

Scan stained smear using oil immersion objective and count a total of 100 or more neutrophils. Record as positive those neutrophils showing formazan deposits. These may occasionally appear diffusely granular, but will predominately occur as large, irregularly shaped, dark purple to black intracytoplasmic inclusions. In counting neutrophils as positive, it is recommended that the stipulations set forth by Feigin³⁴ be followed. These include:

1. The neutrophil must be whole, with cell membrane intact.
2. The neutrophil must be solitary, with no other cell or cellular material (except red blood cells) in contact with it. Neutrophils included in clumps of leukocytes or platelets should not be counted.
3. To be considered positive, the neutrophil must contain formazan deposits as large irregularly shaped, discrete masses.
4. The percent positive of 100 or more neutrophils counted is then recorded.

NOTE: Only neutrophils are counted. Formazan deposits may also occur in monocytes or in clumps of platelets.^{1,3,51,52} As a further refinement, the determination of percent NBT-positive neutrophils can be made concurrently with a total white blood cell count and differential to permit calculation of the absolute number of positive cells. Feigin and coworkers¹⁷ utilized percent NBT-positive and absolute number of NBT-positive neutrophils to develop a nomogram for tentative patient classification. However, use of their published nomogram cannot be recommended unless the accuracy of its application can be demonstrated in your laboratory.

EXPECTED VALUES

Normal Range ¹⁷	2–17%	Positive Cells
Mean	9%	Positive Cells

Most investigators^{1-3,14,18,20-22,40-48,53-55} report mean values for clinically healthy persons to be 10% or less positive (formazan-containing) neutrophils, although some samples may yield values as high as 17%.¹⁷ Most observers agree^{1,2,17-21,40-42} that the percentage of positive neutrophils is usually increased in the presence of bacterial infection, provided leukocytes are metabolically normal. Paterson and Matula² found that all patients with bacteremia exhibit elevated NBT test values.

Normal or Low Values in the Absence of Bacterial Infection are Reported in:

Viral diseases^{1,2,7,17}
Rheumatoid arthritis^{1,5,53}
Pulmonary embolism¹³
Tissue transplant patients^{2,7}
Cancer^{2,18}
Postpartum women^{7,47}
Postoperative patients⁷
Other febrile conditions (or those showing leukocytosis) not of bacterial origin^{1,2,7,15,18}

Normal or Depressed Values in the Presence of Bacterial Infection are Reported in:

Localized infections^{7,13,53,56} (In vitro studies indicate that neutrophil response requires the stimulus to be of adequate strength)
Administration of corticosteroids, phenylbutazone and immuno-suppressive agents^{43,47,49,54}
Antibiotic Therapy where effectiveness may be indicated by a reduction of percent positive, sometimes in less than 6 hours¹⁸
Primary tuberculosis^{7,13}
Metabolic defects of neutrophil function, such as:
Chronic granulomatous disease^{6,7,8,11,42,49}
Neutrophilic deficiencies of myeloperoxidase⁵⁷ or glucose-6-phosphate dehydrogenase⁵⁰
Congenital and acquired agammaglobulinemia⁷
Systemic lupus erythematosus^{1,2,5,18}
Disease states characterized by immune complexes¹⁵
Lipochrome histiocytosis⁷
Chronic myeloid leukemia^{43,44}
Kwashiorkor³⁹
Diabetes⁵⁸
Overwhelming infections^{22,27,54,57}

Elevated Values are Reported in:

Bacterial infections^{1-3,7,13,17,21,33,40-42,53,55,57,58}
Nocardia infections or other systemic fungal diseases^{1,2,57}
Various parasitic infections, including malaria^{2,40,41,48}
Miliary tuberculosis^{7,13}
Tubercular meningitis^{7,13}

Elevated Values in the Absence of Bacterial Infection are Reported In:

Normal infants, less than two months old (also newborn and premature infants)^{7,35,42,55,59}
Pregnancy⁴⁶
Chekiak-Higashi syndrome⁶⁰
Idiopathic myelofibrosis^{15,43}
Osteogenesis imperfecta²¹
Hemophilia²¹
Hodgkin's disease or other lymphomas^{47,61}
Behcet's syndrome⁶²
Inflammatory bowel disease¹⁴
Typhoid/paratyphoid immunization (within a few hours)^{13,33,60}
Streptokinase therapy²²
Viral meningitis⁵⁴
Viral hepatitis⁶³
Post-dialysis²⁰
Patients taking oral contraceptives⁶⁴ (some reports indicate oral contraceptives cause no effect)^{45,46}
Myocardial infarction⁶⁵

Reproducibility studies were performed by the same technologist on blood from 25 patients, employing the described method. Duplicate results, expressed as percent of positive NBT cells, ranged from 2–90%. Use of the statistical method of least squares yielded a correlation coefficient of 0.9834 between duplicates.

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

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