I. PRINCIPLE:

Chronic granulomatous disease of childhood is a lethal X-linked disorder of granulocyte (poly) function. CGD polys can phagocytize microbes, but fail to kill some kinds, specifically catalase-producing organisms like staphylococcus. Normal polys are very effective at making superoxide radicals from H_2O_2 available in phagocytic vacuoles. These superoxide radicals are needed for microbial destruction. CGD polys are less effective in making superoxide radicals. The catalase produced by staphlococcus destroys enough of the H_2O_2 so that CGD polys can't form the superoxide radicals needed to kill microbes. This failure causes recurrent infection and subsequent formation of granulomas. In parallel with their failure to kill microbes, polys from patients with chronic granulomatous disease are unable to reduce nitro-blue tetrazolium (NBT) to formazan during phagocytosis. Normal granulocytes reduce NBT rapidly and become distinctive, degenerative "formazan cells".

II. SPECIMEN:

Blood should be collected in a **red stoppered** tube. If possible record the WBC count and % Neutrophils on the patient's CBC sample. A **normal clot is needed as a control**, to be run along with the patient sample. The normal patient control needs to have a high WBC and increased granulocytes. Check for a CBC sample. The clot should be fresh and held at room temperature for best results. Patient and control clot held for 24 hours at room temperature also gave sufficient results when tested.

Reject: both patient and control samples that have been refrigerated.

III. REAGENTS:

A. Bacterial endotoxin
B. Nitroblue tetrazolium (NBT)
C. Normal human serum
D. Saline
E. Endotoxin coated slides
F. Test media
G. Absolute Methanol
H. Hematek stainer

Stock solution:

1. Bacto, Lipopolysaccharide W, L8274
2. E. coli 026:B6 phenol extraction (2-8°C)

3. Sigma (10 mg bottle)
   Add 10 mg to 200 ml of distilled water. Stable for 1year.
   Working Solution: add 1 ml of stock solution to 99 ml of distilled water.

I. Nitroblue tetrazolium:

1. No. N-6876 crystalline grade III. Add entire 250 mg vial to 89.3 ml normal saline then filter through Nalgene/Sybron 20 micron filtering unit, Cat #120-0020. Alternately, this can be filtered through fluted filter paper (unsterile). Either way it can be frozen in 1-2 ml aliquots at -20° for 1 year.

J. Red top serum and clot with a normal result from a Chemistry rack.

K. Saline

L. Endotoxin coated slides:

1. Frosted end glass slides should be flooded with working endotoxin solution and allowed to air dry <u>completely</u>. Once dry they are ready to be used. Mark an X with pencil on the frosted side that has been flooded with endotoxin, then the tech can tell which side to place the patient and control clot.

M. <u>Test Media</u>:

		<u>Control</u>	<u>NBT</u>
1.	Normal human serum	.5 ml	.5 ml
2.	Normal saline	.9 ml	.3 ml
3.	NBT soln(.28%*)	0	.6 ml
4.	(* final NBT conc. is .12%)		

IV. PROCEDURE:

A. Set incubator at 37^{o}. Thaw NBT reagent and normal serum at room temperature. Moisten humidity chamber.

B. Collect four endotoxin coated slides for each sample to be run (4 slides for patient, 4 slides for normal control). Label two slides for the NBT test media and two slides for the control test media <u>without</u> NBT.

C. Remove clot from red top tube and cut thin slices with a single edged razor blade. Place a slice or two of the clot on each appropriately labeled slide. Using the **glass etcher** make a circle around each clot slice. Alternately place slices side by side

and etch around both. Another technique which yields good results is the maceration of the clot creating a "puddle" of cellular material, etch around this area.

D. Place slides into a moist humidity chamber at 37^{o}C for 25 min. This will allow polys to settle out and stick to the glass surface. Prepare test media while slides are incubating.

E. Remove moist chamber from 37^{o} incubator. Take first slide and gently wash clot off surface with normal saline leaving adherent polys, eos, and monocytes. Disrupt surface of the clot with an applicator stick if necessary. Excess saline can be blotted from the slide. Add two drops of test media to slide and coverslip. Excess media can be blotted from the edges with filter paper. Return slide to moist chamber. Make sure slides are level and that the NBT reagent is dispersed evenly across the slide.

F. Proceed to next slide repeating the same steps outlined in step 6 until all slides are completed.

G. Return moist chamber with slides to 37^{o} incubator for 20 more minutes.

H. Slides are removed, drained, air dried, and fixed for a minute in **absolute methanol** and air dried.

I. Stain **twice** in the Hematek stainer.

J. Mount slides with permount.

V. RESULTS:

The negative <u>control</u> slide will show a carpet of polys, with nuclei more apparent than cytoplasm or cytoplasmic margins. The nuclei stain pink, and may be slightly swollen but normal in shape. Some eos and monocytes should also be present. Should control slides fail to yield expected results, test must be repeated. The <u>NBT</u> slide will, if the donor is normal, show "formazan cells", neutrophils that responded to NBT. These cells have dense, rounded up nuclei and expanded, light blue cytoplasm. Formazan cells look a little like normal monocytes except that the nuclei are rounded up and compacted (they may remind you of blue fried eggs).

If the slide with patient's polys, endotoxin and NBT fails to show numerous formazan cells. The result should be reported as "abnormal, very suspicious for chronic granulomatous disease." If the patient's cells treated this way do assume the blue fried egg appearance, the result is normal.

Slides are left for attending pathologist review and interpretation.

VI. REFERENCE:

1. Gifford RH and Malawista SE: A simple rapid micromethod for detecting chronic granulomatous disease of childhood. J Lab and Clin Med.1970; 75:511 (March).

VII. HISTORY:

H-1 This procedure was written by P. Morris on 10-27-97.
H-2 Revised by Paula Morris on 12/4/10.
H-3 Revised by Paula Morris on 5/5/11.
H-4 This procedure was revised by Susan Richardson 9/2012.
H-5 This procedure was revised by Andrew Link 12/13