Washington University School of Medicine
Neuromuscular Lab
CAP: 1923316
CLIA: 26D0652044
NY: PFI 3499

NADH-TR "Diaphorase" PROTOCOL

PRINCIPLE:

"Diaphorase" is a term given to flavoprotein enzymes that have the property of transferring hydrogen from reduced nicotinamide adenine dinucleotide (NADH) to various dyes. The hydrogen transfer reduces the dye. Usually tetrazolium compounds function as the hydrogen acceptor when diaphorases are being demonstrated histochemically, and the product of the reduction is the water-insoluble formazan pigment. Commonly used tetrazoliums include nitro blue tetrazolium (NBT). Enzymatic activity releases hydrogen from the substrate, and the released hydrogen is transferred to the tetrazolium. With the addition of hydrogen, the tetrazolium is converted to purple-blue formazan pigment marking the site of enzyme activity.

SPECIMEN REQUIRED:

Snap frozen human striated muscle.

Controls:

Stain several different muscles simultaneously. There is normally always enzyme activity present in muscle. The degree and location is interpreted.

METHOD:

Fixation: None. Use snap frozen tissue.
Technique: Cut 10 - 16 micron (12 µm) sections in cryostat from snap frozen muscle. Attach one or more sections to a No. 1½, 22 mm square coverslip.

Equipment:

Ceramic staining rack - Thomas Scientific #8542-E40
Columbia staining dish - Thomas Scientific #8542-C12
Columbia staining dish(jar) - Thomas Scientific #8542-E30
Forceps Latex gloves

Reagents:

Acetone - Baxter #010-4, **FLAMMABLE**
deionized water
Gelatin - 100 bloom -ICN 960317 store at room temperature
Glycerol - Sigma G 8773, store at room temperature **IRRITANT**
Nicotinamide adenine dinucleotide, reduced (NADH)- Sigma N8129
Nitro blue tetrazolium (NBT) - Sigma N6876), store at 0- 5 ^{o} C
Phenol - Fisher A931-1, **CAUSTIC,** Store at room temperature
TRIS base (Tris[hydroxymethyl]aminomethane) - Sigma T6791,
Store at room temperature
TRIS HCl - Sigma T3253, store at room temperature

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Solutions:

I. TRIS BUFFER 0.05 M, pH 7.6 @ room temperature

TRIS HCl	1.43 g
TRIS BASE	0.415 g
deionized water	→250 ml

Store refrigerated, prepare every four to six weeks

II. NADH SOLUTION (8 mg/5 ml)

NADH	80 mg
TRIS BUFFER (solution I)	50 ml

dispense 5 ml aliquots into 13x100 mm glass tubes
cover with caps
Store at -20 ^{o}C

III. ACETONE DESTAINING SOLUTIONS (30%, 60%, 90%)
these solutions need only be approximate

30 % ≅ ACETONE	10 ml
deionized water	20 ml
60 % ≅ ACETONE	20 ml
deionized water	10 ml
90 % ≅ ACETONE	30 ml

IV. Aqueous Mounting Medium (glycerogel)

gelatin (ICN#960317 - 100 bloom	4 g
glycerol	25 ml
phenol (**CAUSTIC !**)	0.5 ml
deionized water	21 ml

1. Dissolve gelatin in boiling water.
2. Cool, but do not allow to solidify.
3. Add phenol and glycerol.
4. Mix well.
5. **Allow air bubbles in mixture to dissipate before using!**

Staining Procedure:

1. Thaw NADH solution.

2. Add 10 mg of NBT to the tube with the NADH solution.(Incubating Solution)

3. Incubate coverslips in a columbia staining dish (Thomas Scientific #8542-C12) for 15 minutes at 37 ^{o}C.

4. Wash with three exchanges of tap, or deionized, H_2O.

5. Prepare approximate solutions of 30, 60 and 90 % acetone using deionized H_2O and remove unbound NBT from the sections with three exchanges each of the acetone solutions in increasing then decreasing concentration. Leave the 90 % acetone covering the sections until a faint purple cloud is seen over the section.

6. Rinse several times with deionized H_2O and then mount the coverslips with the aqueous mounting medium onto a labeled glass slide.

Results:
Blue-Purple formazan precipitate is deposited on mitochondria and sarcoplasmic network. Type I fibers are darker than type II. Walls of blood vessels also are stained. Necrotic muscle fibers lose staining.

REFERENCES:

1. Sheehan, D.C. and Hrapchak, B.B.: THEORY AND PRACTICE OF HISTOTECHNOLOGY Second Edition, Battelle Memorial Institute, 1987.

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