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

AMYLOPHOSPHORYLASE STAIN PROTOCOL

PRINCIPLE:

In the histochemical reaction, phosphorylase acts on the substrate, glucose-1-phosphate and forms, in the presence of a glycogen primer, a polysaccharide composed of α-1,4-glycosyl units. The in vitro reaction of polysaccharide formation is, therefore, the opposite of the in vivo action of glycogen degradation. This happens because the concentration of the substrate is high, and the concentration of the inorganic phosphate is low. The system equilibrium therefore favors glycogen formation. Adenosine-5'-monophosphate functions as an activator.

Exposure of the sections to an iodine solution after incubation results in a varied color formation in the newly formed polysaccharide. A negative reaction is yellow. Unbranched chains of 4 to 6 glucosyl units will give a negative reaction. Polysaccharides of 8 to 12 units gives a reddish color, followed by various transitional colors as the length of the chain increases. Chain lengths of 30 to 35 units give a blue color. The reason the color is blue rather than red-brown is that the polysaccharide formed by the phosphorylase action is not normal glycogen. For glycogen to color a true red-brown, it has to be branched, and branching will only occur if branching enzyme is allowed to act. However, the action of the branching enzyme is eliminated by the inclusion of alcohol in the incubation medium.

QUALITY ASSURANCE:

This enzyme is very labile and the preliminary handling of the specimen is of the utmost importance. Biopsies that have been totally immersed in saline for any length of time or removed with a cautery knife may be compromised. However, there will often be some areas of stain compared to the totally yellow color of a true negative stain.

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 color is interpreted.

METHOD:

Fixation: None, use snap frozen tissue.
Technique: Cut 10 - 16 micron (12 µm) sections in cryostat from snap frozen biopsy. 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:

Absolute alcohol (100% ethanol)
FLAMMABLE, Store at room temp in a flammable cabinet
Acetic Acid (Glacial acetic acid Fisher Scientific A507-500)
Store at room temperature
Adenosine Monophosphate (Adenylic Acid, AMP)
(Sigma A 1752, sodium salt) Store at -20° C, desiccated.
Dextran (Sigma D 1390) avg. mol wt 77000, Store at room temp.
Glucose-1-Phosphate (Sigma G 7000; store at -20° C, desiccated)
Insulin (Sigma I 9276, store at 2 to 8° C)
Potassium Iodide (Sigma P 8256, **TERATOGEN**, Store at room temp.)
Polyvinyl-Pyrrolidone (Sigma PVP 10, store at room temp.)
Reagent alcohol, ACS - histological Fisher A962-4,or HPLC A995
FLAMMABLE, TOXIC, TERATOGENIC
Store at room temp. in flammable cabinet
Sodium acetate anhydrous (Sigma S 8750) Store at room temp.
Sodium Fluoride (Sigma S 1504, store at room temp.)
Sodium Hydroxide (Certified ACS pellets - Fisher S318,
CAUTION CORROSIVE!!

Stock Solutions:

I. **Lugol's Iodine** Solution: (store at room temp. in an amber bottle)

Potassium Iodide (KI)	1 g
dissolve in deionized water	1 ml
when completely dissolved add iodine crystals (I_2)	0.5 g
when iodine is completely dissolved add D.I. H_2O to a final volume	25 ml

II. **Substrate Solution:**

Sodium Acetate Buffer Solution, 0.1 M, pH 5.8 -> 5.9

Sodium acetate anhydrous	1.36 g
Acetic acid glacial	0.12 ml
deionized water	100 ml

Adjust pH with 10 % Acetic Acid or 0.1 N NaOH

Dissolve in order:

Polyvinyl Pyrrolidone	9 g
Sodium Fluoride	180 mg
Dextran	20 mg

Dissolve then add

Glucose-1-Phosphate	1 g
Adenosine Monophosphate	100 mg

Store at -20° C in 10 ml aliquots

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III. **Working Solution** for Stain (**prepare fresh for each assay**)

Thaw Substrate Solution & Bring to room temperature
To a 20 ml glass beaker add
2.0 ml 100 % (absolute) Ethanol
10 ml Substrate Solution
MIX WELL
1 drop Insulin
MIX WELL

Staining Procedure:

1. Place coverslips with sections in a columbia staining dish (Thomas Scientific #8542-C12) at room temperature with the WORKING SOLUTION for a minimum of 60 minutes (overnight is acceptable).
2. Wash with several exchanges of deionized H_2O.
3. Add 50 % ethanol to sections and incubate at room temperature for 10 minutes.
4. Wash sections with 100% ethanol 2 – 3 times &
   Incubate with the 100 % for 10 minutes.
5. Decant the ethanol and air dry at least 10 minutes.
6. Add 6 - 8 drops of Lugol's Iodine to approx. 10 ml deionized water.
7. Add iodine solution to sections until they are dark in color (at least 30 minutes).
8. Remove sections to a ceramic rack.
9. Dip rack with sections rapidly into 95 % alcohol and then
   through 2 rapid dips in 100% alcohol.
10. Leave in air to dry (not necessary to be completely dry)
11. Dip each coverslip individually into xylene.
12. Mount with Permount with a few Iodine crystals dissolved in it.

Results:

Sites of phosphorylase activity -- blue or red-brown deposits of newly synthesized polysaccharide.

REFERENCES:

1. Engel, Andrew M.D. Mayo Muscle Lab, 3/29/01

2. Thompson, Samuel W. *SELECTED HISTOCHEMICAL AND HISTOPATHOLOGICAL METHODS,* Charles C. Thomas, Springfield, IL, 1966.

3. Sheehan, D.C. and Hrapchak, B.B., *THEORY AND PRACTICE OF HISTOTECHNOLOGY,* 2nd Edition; Battelle Memorial Institute, Columbus, OH, 1987.

12/27/2015