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Area	Laboratory-Histology
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Histology Muscle Enzyme - Phosphofructokinase - Royal Oak

Document Type: Procedure

I. PURPOSE AND OBJECTIVE:

The purpose of this document is to provide a procedure for the demonstration of a muscle enzyme, phosphofructokinase. Glycogen Storage Disease is a metabolic disorder that involves the enzymes that regulate glycogen metabolism. The symptoms, severity, and location of the storage of glycogen depend upon which enzyme is affected. Type VII, or Tauri's disease, is caused by a deficiency in muscle phosphofructokinase. Type XII is caused by a deficiency in muscle aldolase. In both, there will be an abnormally high level of glycogen stored in the muscles, because the cells cannot metabolize it.

II. PRINCIPLE:

Aldolase is a catalyst that is involved in the breakdown fructose 1,6-diphosphate into dihydroxyacetone phosphate (ketose) and glyceraldehyde phosphate (aldose). Demonstration of Aldolase staining is carried out concurrently with Phosphofructokinase (PFK) staining to demonstrate the integrity of the metabolic pathway via fructose 1,6-diphosphate. ***Phosphofructokinase*** is the enzyme involved in conversion of ATP and fructose-6-phosphate to fructose 1,6-diphosphate plus ADP plus a hydrogen (H^+).

Phosphofructokinase (tissue) | *Aldolase (Tissue)* |

Fructose-6-phosphate → ADP + Fructose 1,6-diphosphate + H^+ → Dihydroxyacetone Phosphate plus Glyceraldehyde Phosphate

|

ATP

The Hydrogen (H^+) reduces the tetrazolium salt, nitro blue tetrazolium (NBT), forming a highly colored formazan dye which is finely granular blue.

H^+ + Nitro blue tetrazolium (NBT) → reduced tetrazolium → formazan (blue)

III. SPECIMEN COLLECTION AND HANDLING:

A. Fixation
    1. Unfixed tissue that has been frozen.

B. Processing
    1. Fresh tissue.
    2. No processing.

C. Section Thickness
    1. Cut frozen sections at 10µ.

D. Slide Drying
    1. None.

E. Type of slide
    1. Plus slides.
    2. Use 2 patient slides; write “Negative” on one slide.

IV. REAGENTS:

A. **0.2 M Hydrochloric Acid**

Hydrochloric acid, conc (HCl)	**1.7 ml**
Distilled water	**100.0 ml**

Slowly add hydrochloric acid, drop by drop, to water. Stir. Store at room temperature; stable for months.

B. **0.1 M Hydrochloric Acid**

Hydrochloric acid, conc (HCl)	**1.7 ml**
Distilled water	**200.0 ml**

Slowly add hydrochloric acid, drop by drop, to water. Stir; store at room temperature; stable for months.

C. **0.1 M Sodium Hydroxide**

Sodium hydroxide (NaOH)	**4.0 gm**
Distilled water	**1000.0 ml**

Dissolve together; store at room temperature; stable for months.

D. **0.2 M Sodium Cacodylate stock (Same as “Aldolase Stock”)**

Sodium Cacodylate, sodium salt, trihydrate (Sodium arsenate) ($C_2H_6AsNaO_2i3H_2O$) **4.28 gm**

Distilled water

100.0 ml

Dissolve sodium cacodylate and water together; stable for months.

E. **Cacodylate Buffer, pH 7.0**

0.2 M Sodium Cacodylate stock	**25.0 ml**
0.2 M Hydrochloric Acid	**3.2 ml**
Distilled water	**71.8 ml**

JUST BEFORE USE, mix together. Adjust pH to 7.0 with either 0.1 M Sodium hydroxide or 0.1 M hydrochloric acid. Warning – pH jumps quickly as it nears 7.0. Dilute 0.1 M NaOH or HCl solutions, if necessary.

F. **10% Calcium Chloride**

Calcium chloride ($CaCl_2i2H_2O$)	**10.0 gm**
Distilled water	**100.0 ml**

Mix together. Use to make the Formol-Calcium.

G. **Formol-Calcium**

40% Formaldehyde (HCHO)	**100.0 ml**
Distilled water	**900.0 ml**
10% Calcium Chloride	**100.0 ml**

Mix together in hood to avoid formaldehyde fumes. Store at room temperature; stable for years.

H. **Incubating Solution**

Fructose-6-phosphate, disodium salt (Sigma F-3627)	**0.16 gm**
Distilled water	**2.0 ml**

JUST BEFORE USE, dissolve together. Then add:

0.2 M Sodium Cacodylate buffer, pH 7.0	**18.0 ml**
Nicotinamide Adenine Dinucleotide (NAD) (Sigma N-1511)	**0.04 gm**
Nitro Blue Tetrazolium (NBT) (Sigma N-6876)	**0.01 gm**
Adenosine Triphosphate, disodium salt (ATP) (Sigma A-7699)	**0.02 gm**
Magnesium chloride, anhydrous ($MgCl_2$) (1-2 small crystals)	**0.004 gm**

JUST BEFORE USE, mix together in first part of incubating solution. All NBT may not dissolve at room temperature but will dissolve upon incubation. Adjust pH to 7.0 with 0.1 M Hydrochloric acid or 0.1 M Sodium Hydroxide. *Warning – pH jumps quickly as it nears 7.0. Dilute 0.1 M Hydrochloric acid or 0.1 M Sodium Hydroxide, if necessary.* Use immediately.

I. **Negative Control Incubating Solution**

0.2 M Sodium Cacodylate buffer	**18.0 ml**
Distilled water	**2.0 ml**
Nicotinamide Adenine Dinucleotide (NAD) (Sigma N-1511)	**0.04 gm**
Nitro Blue Tetrazolium (NBT) (Sigma N-6876)	**0.01 gm**
Adenosine Triphosphate, disodium slat (ATP) (Sigma A-7699)	**0.02 gm**
Magnesium chloride, anhydrous ($MgCl_2$) (1-2 small crystals)	**0.004 gm**

JUST BEFORE USE, mix together in first part of incubating solution. All NBT may not dissolve at room temperature but will dissolve upon incubation. Adjust pH to 7.0 with 0.1 M Hydrochloric acid or 0.1 M Sodium Hydroxide. *Warning – pH jumps quickly as it nears 7.0. Dilute 0.1 M Hydrochloric acid or 0.1 M Sodium hydroxide, if necessary.* Use immediately.

V. EQUIPMENT:

A. Mettler balance
B. 37°C oven
C. pH meter

VI. SUPPLIES:

A. Erlenmeyer flasks
B. Graduated cylinders
C. Funnel
D. Coplin jars
E. Pipets

VII. QUALITY CONTROL(QC):

Frozen section of muscle. Normal muscle, frozen section, may be used as a positive control section for aldolase.

VIII. SPECIAL SAFETY PRECAUTIONS:

A. Hydrochloric Acid
    1. Is corrosive.
    2. Store in an acid cabinet.
B. Sodium Hydroxide
    1. Is corrosive.
C. Sodium Cacodylate
    1. Is toxic, mutagenic, and can cause gastro-intestinal damage resulting in vomiting and diarrhea, and general vascular collapse leading to shock, coma and death.
    2. Muscular cramps, facial edema, and cardio-vascular reactions are also known to occur following oral exposure.
D. Fructose-6-Phosphate
    1. Is an irritant.
E. Nitroblue Tetrazolium (NBT)
    1. Is an irritant.
F. Nicotinamide Adenine Dinucleotide (NAD)
    1. Is an irritant.
G. Adenosine Triphosphate (ATP)

1. Is an irritant.

H. Magnesium Chloride
    1. Is an irritant and is hygroscopic.

I. Calcium Chloride
    1. Is an irritant and is hygroscopic.

J. Formaldehyde
    1. Is a poison.
    2. May be fatal or cause blindness if swallowed.
    3. Cannot be made non-poisonous.
    4. Possible cancer hazard. Irritating to eyes, skin, and respiratory tract.
    5. Can cause severe eye burns.

IX. LIMITATIONS:

A. Procedure is very pH sensitive. Care must be taken to control pH.

B. The pH tends to rise quickly, so dilution of the 0.1 N Hydrochloric acid may be necessary.

C. Fiber-typing must be based on color differences rather than on intensity differences.

D. Dithiothreitol, NBT, and NADH MUST be kept frozen for storage to prevent deterioration.

E. Not all the NBT will dissolve at room temperature. It will finish dissolving during the 37°C. incubation.

F. Make certain using **NAD**, ***NOT NADH***, reduced.

G. It is essential to incubate adjacent patient sections in “negative control” incubating solution, as the background on both normal and PFK negative patients will be a pale blue staining.

H. You must pH Solution “A” **BEFORE** the addition of dithiothreitol. The dithiothreitol is harmful to pH probes.

I. This enzyme is very sensitive to fixation. Avoid all types of fixatives.

X. PROCEDURE:

Step	Action	Time	Notes
1	Take 2 patient slides. On one, write “Negative”. This will be the negative substrate control.		
2	Pour incubating solution into a plastic slide mailer and place patient slide into solution.		Cover to prevent evaporation
3	Pour negative control incubating solution into a slide mailer and place patient "negative" control slide into the solution.		Cover to prevent evaporation

4	Incubate both slides in their own solution.	1 hour	Must be made fresh just before use. Incubate in 37°C oven. Incubation time may range from 30-90 minutes.
5	Rinse with distilled water.	1 minute	Rinse carefully, as sections have a tendency to fall off.
6	Combine slides together.		
7	Fix in Formol-Calcium.	10 minutes	
8	Rinse in distilled water.	1 minute	
9	Mount with Apathy Gum Syrup. Seal coverslip using clear fingernail polish.		

XI. RESULTS:

A. Phosphofructokinase (PFK) - **blue**
(Found in cytoplasm of muscle fibers)
(Type I may be darker than Type II, but cannot be used for fiber typing)

B. Deficiency of PFK - **pale staining**

C. Background, normal and PFK deficient - **pale staining**

D. Slides in “Negative Control Incubating Media” - **pale staining**

XII. REFERENCES:

A. Bancroft JD, Stevens A: Theory and Practice of Histological Techniques, 3rd ed. New York, NY. Churchill Livingstone, 1990.

Approval Signatures

Step Description	Approver	Date
Medical Director	Kurt Bernacki: System Med Dir, Surgical Path	1/18/2023
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Applicability

Royal Oak