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

ADENOSINE TRIPHOSPHATASE (ATP) STAINING PROTOCOL

PRINCIPLE:

The calcium method for ATPase demonstration, employing solutions of different pH values, is used primarily to distinguish muscle fiber types. Muscle fibers may be broadly categorized as type 1 ("slow, red muscle, oxidative") and type 2 ("fast, white muscle, glycolytic"). Type 2 muscle fibers are further subdivided as 2a (glycolytic), 2b (glycolytic/oxidative), and 2c which are immature or changing types due to reinnervation or injury. This stain is believed to work is as follows: The preincubation pH inactivates the myosin-ATPase enzyme of specific fiber types. The remaining active enzyme is attached to a calcium atom which is replaced by a cobalt which is precipitated as a black insoluble compound by the ammonium sulfide.

QUALITY ASSURANCE:

This is a complicated stain. Several areas require attention to achieve a good fiber type differentiation.

(1) pH of all solutions.
(2) Timing.
(3) pH solutions: *Sodium hydroxide, should be < 2 months old (especially the 0.1 N).*
(4) *Stock ammonium sulfide must be yellow*. As it ages or oxidizes, it becomes progressively red to the point where it cannot be used.

SPECIMEN REQUIRED:

Snap frozen human striated muscle.

METHOD

Fixation: None. Use snap frozen tissue.

Technique: Cut 10 - 16 micron (14 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:

Adenosine triphosphate, disodium salt - Sigma A2383
Ammonium sulfide (light solution, original stock: concentration = 20 %)
NOTE: if ammonium sulfide is a dark yellow, it must be replaced.
– Sigma A1952
COMBUSTIBLE,ODORIFEROUS, USE IN HOOD
Calcium Chloride, anhydrous - Sigma C4901,
Store at room temperature
Canada Balsam, filtered neutral, Fisher B10-100
Cobalt chloride hexahydrate, ACS - Sigma C3169 -
TOXIC, MUTAGENIC
deionized water
Hydrochloric acid, ACS - Fisher A144-500
CORROSIVE, store at room temperature
Sodium acetate, trihydrate - Sigma S9513, Store at room temperature
Reagent alcohol, ACS - histological Fisher A962-4 or HPLC A995,
FLAMMABLE, TOXIC, TERATOGENIC,
Store at room temperature in flammable cabinet
Sodium barbital (5,5' dietyl barbituric acid) - Sigma B0500,
NARCOTIC, TOXIC,CONTROLLED SUBSTANCE,
Store at room temperature
Sodium Hydroxide , Certified ACS pellets - Fisher S318,
CAUTION CORROSIVE!!
Xylenes - Fisher #HC700-1GAL, **FLAMMABLE**,
Store at room temperature in flammable cabinet.

Solutions:

I. 0.1 M Sodium Barbital Solution
(5.15 gm barbital powder (room temp.)+ deionized $H_2O \rightarrow$ 250 ml)
store at room temperature

2. 0.18 M Calcium Chloride
(2.65 g $CaCl_2 \cdot 2H_2O$ + deionized water $\rightarrow$ 100 ml)
store at room temperature

3. 1 % w/v Calcium Chloride
($\cong$ 5 g $CaCl_2 \bullet 2H_2O$ + deionized water $\rightarrow$ 500 ml)
store at room temperature

4. 2 % w/v Cobalt Chloride
($\cong$ 4 g $CaCl_2 \bullet 6H_2O$ + deionized water $\rightarrow$ 200 ml)
store at room temperature

5. Barbital Acetate Solution

sodium barbital (sodium barbiturate)	1.47 g
sodium acetate ($NaC_2H_3O_2 \bullet 3H_2O$)	0.97 g
deionized water $\rightarrow$ final volume of 50 ml	

6. 1 N Sodium Hydroxide
   4 g NaOH + deionized water → 100 ml
   store at room temperature

7. 0.1 N Sodium Hydroxide
   1 ml 1 N NaOH + 9 ml deionized water
   store at room temperature

8. 1 N Hydrochloric Acid
   10.4 ml concentrated (≅ 12 N) H Cl added to deionized water → 125 ml
   store at room temperature

9. 0.1 N Hydrochloric Acid
   10 ml 1 N H Cl to 90 ml deionized water
   store at room temperature

10. Pre-Incubating Solutions **(prepare fresh for each stain)**

A). **"9.4 ATP"**
(TYPE 2 FIBERS DARK, TYPE 1 FIBERS LIGHT)

2.0 ml 0.1 M Sodium Barbital
2.0 ml 0.18 M Calcium Chloride
6.0 ml deionized water

adjust pH between 9.8 → 10.0 just prior to use with a few drops of 0.1 N NaOH (muscle other than human usually requires a pH of ~ 10.2)

B). **"4.6 ATP"**
(TYPE 1 FIBERS DARKEST, TYPE 2b FIBERS INTERMEDIATE, TYPE 2a LIGHTEST)

2.5 ml Barbital Acetate Solution
5.0 ml 0.1 N HCl
2.0 ml deionized water

adjust pH between 4.57 → 4.60 (4.6) just prior to use with a few drops of 1 N HCl (muscle other than human usually requires a pH of ~ 4.5)

C). **"4.3 ATP"**
(TYPE 1 FIBERS DARKEST, TYPE 2c FIBERS INTERMEDIATE, TYPE 2a&b LIGHTEST)

2.5 ml Barbital Acetate Solution
5.0 ml 0.1 N HCl
2.0 ml deionized water

adjust pH between 4.30 → 4.31 just prior to use with a few drops of 1 N HCl (muscle other than human usually requires a pH of ~ 4.2)

11. **ATP Incubating Solution** (volume here is sufficient for three (3) staining jars)

60 mg ATP powder (disodium salt, Sigma # A2383)
7.0 ml 0.1 M Sodium Barbital
20.0 ml deionized water
3.0 ml 0.18 M Calcium Chloride

add the calcium chloride last to prevent precipitation of ATP !

prepare just prior to use and adjust pH to 9.4 (9.35 - 9.45) with a few drops of 1N NaOH and 0.1 N NaOH

DO NOT ALLOW THE pH TO BECOME TOO ALKALINE (> 10.0) AS THIS WILL CAUSE THE ATP TO PRECIPITATE NECESSITATING STARTING OVER!

12. Alcohol 50 %

reagent alcohol	~50 ml
deionized water	~50 ml

13. Alcohol 70 %

reagent alcohol	~70 ml
deionized water	~30 ml

14. Alcohol 80 %

reagent alcohol	~80 ml
deionized water	~20 ml

15. Alcohol 95 %

reagent alcohol	~95 ml
deionized water	~ 5 ml

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Staining Procedure

1. Place one coverslip for each biopsy in a separate, labeled columbia staining dish (Thomas Scientific #8542-C12) for each pre-incubating solution.

2. Incubate in the 4.7 and 4.3 solutions for exactly five (5) minutes at room temperature. The 9.4 solution should be added for fifteen (15) minutes at room temperature.

3. After the appropriate pre-incubation time periods, pour out the solution.

4. Pour the **ATP solution** into the staining jar:
   25 minutes for the 4.6 and 4.3 ATP stains and
   15 minutes for the 9.4 stain

5. Wash each staining jar with three (3) changes of 1% Calcium Chloride for a total of approximately ten (10) minutes.

6. Add 2% Cobalt Chloride to each jar for ten (10) minutes.

7. Wash with three (3) to five (5) changes of an approximately 1:20 solution of 0.1M Sodium Barbital (~ 5 ml 0. 1 M Sodium Barbital + deionized water $\rightarrow$ ~ 100 ml).
   Note: the initial wash should turn a faint blue in color.

8. **THIS STEP SHOULD BE DONE IN A FUME HOOD!! NOXIOUS & TOXIC FUMES!!**
   A. Prepare 10 ml for each staining jar
      $\cong$ 1 % v/v solution of ammonium sulfide (0.2 ml stock NH_4SO_2 + 9.8 ml D.I.H_2O. **NOTE: if the NH_4SO_2 is a dark yellow and there is a precipitate after adding water, the stain may not work and new NH_4SO_2 must be used**.
   B. Add this solution to each jar for at least 3-5 mins. (sections will appear very dark)
   C. Rinse in the fume hood with approximately 3 - 5 changes of tap water.

9. Transfer the coverslips with the stained sections immediately to a porcelain rack, cleaning the back side of the coverslip with a cotton tipped swab if necessary.

10. Dehydrate in ascending alcohols (50%,70%,80%,95% x 2, 100% x 2) in columbia staining dish(jar) - Thomas Scientific #8542-E30 and clear with at least two changes of xylene, also done in columbia staining dish(jar) - Thomas Scientific #8542-E30.

11. Mount coverslips onto labeled glass slides with **CANADA BALSAM ONLY!**
    NOTE: With other mounting mediums, color fades rapidly over a period of weeks.

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Results

ATPase pH	TYPE 1	TYPE 2A	TYPE 2B	TYPE 2C
9.4	light (+1)	dark (+3)	dark (+3)	dark (+2→ +3)
4.6	dark (+3)	light (0)	intermediate (+1→ +2)	intermediate (+1→ +2)
4.3	dark (+3)	light (0)	light (0)	intermediate (+1→ +2)

NOTE: Muscle kept at room temperature for > 12 hours before freezing will have no 2A fibers (absent staining) at ATPase pH 4.6.

REFERENCES

1. Brooke, M.H. and Kaiser, K.K., *Arch. Neurol.,* **23**: 369 - 379, Oct. 1970.

2. Brooke, M.H. and Kaiser, K.K., *J. Histochem. Cytochem.,* **18**: 670 - 672, 1970.

3. Dubowitz, V. and Brooke, M.H. **MUSCLE BIOPSY: A MODERN APPROACH**, W.B. Saunders Co., Ltd, London, 1973.

4. Sheehan and Hrapchak, **HISTOTECHNOLOGY**, 2nd Edition; Batelle Press, Columbus, 1987.

4. Planer, G.J., Pestronk, A., et. al., *Muscle & Nerve,* Feb. 1992.

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