

Myosin ATPase Stain - Fiber Typing in SKM

modified from Neuromuscular Clinical Laboratory, Neurology, WUMS

Principle:

The calcium method for ATPase demonstration, employing solutions of different pH values, have been 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"), and 2c which may be fibers that are changing type due to disease or injury. The way this stain is believed to work is as follows. The pre-incubation 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 and finally precipitated as a black insoluble compound by the ammonium sulfide.

Quality Assurance:

This is a complicated stain and there are several areas in which one needs to be careful in order to achieve a good differentiation.

- 1) The pH of all the solutions is critical.
- 2) Timing is crucial.
- 3) Another source for inadequate differentiation is the pH solutions, particularly the sodium hydroxide, which should not be more than 2 months old (especially the 0.1N).
- 4) The stock ammonium sulfide must still be yellow. As it ages or oxidizes, it becomes more red to the point where it cannot be used.

Specimen Required:

Snap freeze tissue using the isopentane freezing method described in the separate protocol. Frozen muscle tissue should be cut in cross section (10-12 μm thickness) and attached to slides. Slides can be stained the day of or the day after sectioning. Keep in 4°C overnight if necessary.

Reagents:

- Adenosine Triphosphate, disodium salt – Sigma A2383
- Ammonium Sulfide (light solution, original stock concentration = 21%) – Fisher A705-225 **Combustible, odoriferous, USE IN HOOD**
- Calcium Chloride, anhydrous – Sigma C4901
- Canada Balsam, filtered neutral – Fisher B10-100 (Thin with a bit of toluene)
- Cobalt Chloride hexahydrate, ACS – Sigma C3169 **Toxic, Mutagenic**
- Hydrochloric Acid, ACS – Fisher A144-500 **Corrosive**
- Sodium Acetate, trihydrate – Sigma S9613

- Reagent alcohol, ACS, histological – Fisher A962-4 **Flammable, Toxic, Teratogenic**
- Sodium Barbital (5,5' diethyl barbituric acid) – Sigma B0500 **Narcotic, Toxic, Controlled Substance**
- Sodium Hydroxide, Certified ACS pellets – Fisher S318 **Corrosive**
- Xylenes – Fisher HC700 **Flammable**

Solutions (make in advance):

1. 0.1M Sodium Barbital Solution
(10.3g bartibal, q.s. to 500mL)
Store at Room Temperature

2. 0.18M Calcium Chloride
(2.65g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, q.s. to 100mL)
Store at Room Temperature

3. 1% w/v Calcium Chloride
(~5g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, q.s. to 500mL)
Store at Room Temperature. This goes bad often. Discard if trouble with stains.

4. 2% w/v Cobalt Chloride
(~4g $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, q.s. to 200mL)
Store at Room Temperature

5. Barbital Acetate Solution
Sodium Barbital 2.94g
Sodium Acetate 1.94g
H₂O q.s. to 100mL

6. 1N Sodium Hydroxide
(4g NaOH, q.s. to 100mL)
Store at Room Temperature

7. 0.1N Sodium Hydroxide
(1mL 1N NaOH + 9mL H₂O)
Store at Room Temperature

8. 1N Hydrochloric Acid
(41.3mL concentrated (12.1N) HCl, q.s. to 500mL)
Store at Room Temperature

9. 0.1N Hydrochloric Acid
(1mL 1N HCl + 9mL H₂O)

Store at Room Temperature

Solutions (prepare fresh):

1. Pre-Incubation Solutions (**prepare fresh for each stain**) (Note these pH were determined using the Huss lab pH meter.)

A) "10.20 ATP"

(Type II fibers dark, Type I fibers light)

10.0 mL 0.1M Sodium Barbital

30.0 mL H₂O

10.0 mL 0.18M Calcium Chloride

adjust pH just prior to use with a few drops of 0.1N NaOH

B) "4.53 ATP"

(Type I fibers darkest, Type IIb fibers intermediate, Type Ia fibers lightest)

12.5 mL Barbital Acetate Solution

2.5 mL 1N HCl

32.5 mL H₂O

adjust pH just prior to use with a few drops of 1N HCl

C) "4.31 ATP"

(Type I fibers darkest, Type IIc fibers intermediate, Types IIa & IIb lightest)

12.5 mL Barbital Acetate Solution

2.5 mL 1N HCl

32.5 mL H₂O

adjust pH just prior to use with a few drops of 1N HCl

2. ATP Incubating Solution (Volume is for 3 jars or 1 box)

0.36g ATP

48 mL 0.1M Sodium Barbital Solution

114 mL H₂O

18 mL 0.18M CaCl₂ Solution

Adjust pH just prior to use to 9.4 (9.35-9.45) with a few drops of 1N NaOH

Add the CaCl₂ Solution last to prevent precipitation of the ATP!!

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

3. Alcohol Solutions:

Prepare solutions of 50, 70, 80 and 95% alcohol using reagent alcohol and ddH₂O. You will need about 200 mls of 50, 70 and 80%, and about 400 mls of 95 and 100%. Store in Flammable cabinet.

Staining Procedure:

1. Place one slide from each sample in a separate, labeled Coplin jar for each pre-incubation solution.
2. Incubate in the 4.53 and 4.31 solutions for exactly 5 minutes at room temperature. The 10.20 solution should be incubated for 15 minutes (not so critical to be exact). Start all 3 at the same time.
3. After the appropriate pre-incubation time period, pour out the solution and rinse one time with ddH₂O.
4. Pour the ATP solution into the staining jar. Leave for
 - a. 25 minutes for the 4.53 & 4.31 stains and
 - b. 15 minutes for the 10.20 stain.
5. Wash each staining jar with 3 changes of 1% Calcium Chloride Solution for a total of about 10 minutes (~3 minutes each).
6. Add 2% Cobalt Chloride to each jar for 10 minutes.
7. Wash with 3-5 changes of an approximately 1:20 solution of 0.1M Sodium Barbital (~10mL of 0.1M Sodium Barbital + ddH₂O to 200mL for each change)
Note: the initial wash should turn a faint blue in color.
8. Wash with 5 exchanges of ddH₂O.
9. **THIS STEP MUST BE DONE IN A FUME HOOD! NOXIOUS & TOXIC FUMES!**
 - a. Prepare 50 mL for each Coplin jar. ~2% v/v solution of ammonium sulfide (1mL stock NH₄SO₂ + 49mL ddH₂O).
 - b. Add this solution to each jar for 20-30 seconds (or longer, no matter. Sections will appear very dark.)
 - c. Rinse in the fume hood with approximately 5 changes of tap water. Leave in water until ready to dehydrate.
10. Transfer the slides with the stained sections to the slide holder, cleaning the back sides of each with a cotton-tipped swab if necessary.

11. Dehydrate in ascending alcohols (50%, 70%, 80%, 95%x2, 100%x2) and clear with at least 2 changes of xylene.
12. Mount coverslips onto labeled slides with **CANADA BALSAM ONLY!**

Results:

| Pre-Inc. pH | Type I | Type IIa | Type IIb | Type IIc |
|-------------|--------|----------|--------------|--------------|
| 10.20 | light | dark | dark | dark |
| 4.53 | dark | light | intermediate | intermediate |
| 4.31 | dark | light | light | intermediate |

References:

1. Brooke MH and Kaiser KK *Arch. Neurol.*, 23: 369-379, 1970.
2. Brooke MH and Kaiser KK *J. Histochem. Cytochem.*, 18: 670-672, 1970.
3. Dubowitz V and Brooke MH *Muscle Biopsy: A Modern Approach*, W.B. Saunders Co., Ltd., London, 1973.
4. Planer GJ, Pestronk A, et al., *Muscle and Nerve*, 1992.