Enzymatic Assay of ADENOSINE 5'-TRIPHOSPHATASE
(EC 3.6.1.3)
From Dog and Rabbit Kidney

PRINCIPLE:

\[ \text{ATP} + \text{H}_2\text{O} \xrightarrow{\text{ATPase}} \text{ADP} + \text{P}_i \]

Abbreviations used:
ATPase = Adenosine 5'-Triphosphatase
ATP = Adenosine 5'-Triphosphate
ADP = Adenosine 5'-Diphosphate
P_i = Inorganic Phosphate

CONDITIONS: \( T = 37^\circ\text{C}, \text{pH 7.4, } A_{660\text{nm}}, \text{Light path} = 1 \text{ cm} \)

METHOD: Colorimetric

REAGENTS:

A. 50 mM Tris HCl Buffer, pH 7.4 at 37°C
   (Prepare 100 ml in deionized water using Trizma Base. Adjust to pH 7.4 at 37°C with 1 M HCl.)

B. 15 mM Ouabain Solution
   (Prepare 10 ml in deionized water using Ouabain, Octahydrate.)

C. 600 mM Potassium Chloride Solution (KCl)
   (Prepare 10 ml in deionized water using Potassium Chloride.)

D. 2.5 M Sodium Chloride Solution (NaCl)
   (Prepare 10 ml in deionized water using Sodium Chloride.)

E. 90 mM Magnesium Chloride Solution (MgCl_2)
   (Prepare 10 ml in deionized water using Magnesium Chloride Hexahydrate.)
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REAGENTS: (continued)

F. 80 mM Adenosine 5'-Triphosphate (ATP)
(Prepare 10 ml in deionized water using Adenosine 5'-Triphosphate, Tris.
Adjust to pH 7.4 at 37°C with 1 M Tris.)

G. 10% (w/v) Ammonium Molybdate Solution
(Prepare 25 ml in 10 N H₂SO₄ using Molybdic Acid, Ammonia Tetrahydrate Salt.)

H. Taussky-Shorr Color Reagent (TSCR)
(Prepare by adding 10 ml of Reagent G to 70 ml of deionized water. Add 5 g of Ferrous
Sulfate, Heptahydrate. Bring the volume to 100 ml with deionized water.)

I. 20% (w/v) Trichloroacetic Acid (TCA)
(Prepare 100 ml in deionized water using Trichloroacetic Acid, 6.1 N, approximately 100%
(w/v).)

J. Phosphorus Standard (P Std)
(Use Phosphorus Standard Solution. The concentration is 20 µg/ml,
0.645 μmoles/ml.)

K. Adenosine 5'-Triphosphatase Enzyme Solution
(Immediately before use, prepare a solution in cold deionized water containing 4 - 5 units/ml.)

PROCEDURE:

Step 1:

Pipette (in milliliters) the following reagents into suitable containers:

<table>
<thead>
<tr>
<th>Reagent A (Buffer)</th>
<th>Test 1</th>
<th>Blank 1</th>
<th>Test 2</th>
<th>Blank 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent B (Ouabain)</td>
<td>0.90</td>
<td>0.90</td>
<td>0.90</td>
<td>0.90</td>
</tr>
<tr>
<td>Reagent C (KCl)</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Reagent D (NaCl)</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Reagent E (MgCl₂)</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Reagent K (Enzyme)</td>
<td>0.10</td>
<td>------</td>
<td>0.10</td>
<td>------</td>
</tr>
<tr>
<td>Deionized Water</td>
<td>0.20</td>
<td>0.20</td>
<td>0.10</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Mix and equilibrate for 5 minutes at 37°C. Then add:

<table>
<thead>
<tr>
<th>Reagent F (ATP)</th>
<th>Test 1</th>
<th>Blank 1</th>
<th>Test 2</th>
<th>Blank 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
</tr>
</tbody>
</table>
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PROCEDURE:  (continued)

Mix and incubate at 37°C for exactly 5 minutes. Then add:

<table>
<thead>
<tr>
<th></th>
<th>Test 1</th>
<th>Blank 1</th>
<th>Test 2</th>
<th>Blank 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent I (TCA)</td>
<td>1.50</td>
<td>1.50</td>
<td>1.50</td>
<td>1.50</td>
</tr>
</tbody>
</table>

Immediately mix by inversion. Then add:

<table>
<thead>
<tr>
<th></th>
<th></th>
<th>Test 2</th>
<th>Blank 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent K</td>
<td>------</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>(Enzyme Solution)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mix by inversion and then centrifuge in a clinical centrifuge for 3 minutes to clarify.

Step 2:

Pipette (in milliliters) the following into suitable tubes:

<table>
<thead>
<tr>
<th></th>
<th>Test 1</th>
<th>Blank 1</th>
<th>Test 2</th>
<th>Blank 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent H (TSCR)</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td>Test Supernatant</td>
<td>1.00</td>
<td>------</td>
<td>1.00</td>
<td>------</td>
</tr>
<tr>
<td>Blank Supernatant</td>
<td>------</td>
<td>1.00</td>
<td>------</td>
<td>1.00</td>
</tr>
<tr>
<td>Deionized Water</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Mix and incubate at 25°C for 10 minutes. Read the A_{660nm} for both Tests and Blanks.

Standard Curve:

Prepare a standard curve by pipetting the following into suitable tubes (milliliters).

<table>
<thead>
<tr>
<th></th>
<th>Std 1</th>
<th>Std 2</th>
<th>Std 3</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent H (TSCR)</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td>Reagent J (P Std)</td>
<td>0.25</td>
<td>0.50</td>
<td>1.00</td>
<td>------</td>
</tr>
<tr>
<td>Reagent I (TCA)</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>Deionized Water</td>
<td>1.25</td>
<td>1.00</td>
<td>0.50</td>
<td>1.50</td>
</tr>
</tbody>
</table>

Mix and incubate at 25°C for 10 minutes. Read the A_{660nm} for each standard using a suitable spectrophotometer.
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CALCULATIONS:

Standard Curve:

\[ \Delta A_{660\text{nm}} \text{ Standard} = A_{660\text{nm}} \text{ Standard} - A_{660\text{nm}} \text{ Standard Blank} \]

Prepare a standard curve by plotting \( \Delta A_{660\text{nm}} \text{ Standard} \) vs Phosphate concentration.

Sample Determination:

\[ \Delta A_{660\text{nm}} \text{ Test}_1 = A_{660\text{nm}} \text{ Test}_1 - A_{660\text{nm}} \text{ Blank}_1 \]

\[ \Delta A_{660\text{nm}} \text{ Test}_2 = A_{660\text{nm}} \text{ Test}_2 - A_{660\text{nm}} \text{ Blank}_2 \]

Determine the micromoles of Phosphate liberated for each Test using the standard curve.

Units/ml enzyme = \( \frac{(\mu\text{moles of Phosphate released})(3.0)(df)}{(5)(0.1)(1.0)} \)

1.0 = Aliquot (in milliliter) of Test Supernatant used in Step 2
3 = Total volume (in milliliters) of assay (Step 1)
5 = Time of assay (in minutes) as per the Unit Definition
0.1 = Volume (in milliliter) of enzyme used in Step 1

Units/mg solid = \( \frac{\text{units/ml enzyme}}{\text{mg solid/ml enzyme}} \)

Units/mg protein = \( \frac{\text{units/ml enzyme}}{\text{mg protein/ml enzyme}} \)

Test 1 = ATPase, activated (Na, K, Mg)
Test 2 = ATPase, not Ouabain sensitive
Ouabain sensitive = Test 1 - Test 2

UNIT DEFINITION:

One unit will liberate 1.0 \( \mu \text{mole} \) of inorganic phosphorus from ATP per minute at pH 7.4 at 37°C in the presence of Na\(^+\), K\(^+\), and Mg\(^{2+}\).
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FINAL ASSAY CONCENTRATION:

In a 1.50 ml reaction mix, the final concentrations are 30 mM Tris, 3 mM magnesium chloride, 5.3 mM adenosine 5'-triphosphate, 167 mM sodium chloride, 20 mM potassium chloride, 1 mM ouabain (when present), and 0.1 - 0.2 unit ATPase.

REFERENCES:


NOTES:

1. These units are the fully active (Test 1) units.

2. This assay is based on the cited references.