Enzymatic Assay of CREATININASE
(EC 3.5.2.10)

PRINCIPLE:

Creatinine + H$_2$O $\xrightarrow{\text{Creatininase}}$ Creatine

CONDITIONS: $T = 37^\circ$C, pH = 6.5, $A_{525nm}$, Light path = 1 cm

METHOD: Colorimetric

REAGENTS:

A. 300 mM Potassium Phosphate Buffer, pH 6.5 at 37°C
   (Prepare 100 ml in deionized water using Potassium Phosphate, Monobasic, Anhydrous, Sigma Prod. No. P-5379. Adjust to pH 6.5 at 37°C with Reagent B.)

B. 300 mM Potassium Phosphate Solution
   (Prepare 100 ml in deionized water using Potassium Phosphate, Dibasic, Trihydrate, Sigma Prod. No. P-5504.)

C. 10 mM Potassium Phosphate Buffer, pH 8.0 at 37°C
   (Enzyme Diluent)
   (Prepare 25 ml in deionized water using Reagent A. Adjust to pH 8.0 at 37°C with Reagent B.)

D. 0.1 mM Mercury Chloride and 189 mM Sodium Carbonate Solution (Stop Rgt)
   (Prepare 100 ml in deionized water using Mercuric Chloride, Sigma Prod. No. M-1136 and Sodium Carbonate, Anhydrous, Sigma Prod. No. S-2127.)

E. 139 mM a-Naphthol Solution (a-Naphthol)
   (Prepare 100 ml in Reagent F using a-Naphthol, Sigma Prod. No. N-1000.)

F. 95% (v/v) Ethanol (EtOH)
   (Prepare 100 ml in deionized water using 200 Proof USP Ethyl Alcohol, available from Quantum Chemical Company.)
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REAGENTS:  (continued)

G. 300 mM Sodium Hydroxide and 300 mM Sodium Carbonate Solution (Alk Soln)  
(Prepare 100 ml in deionized water using Sodium Hydroxide, Anhydrous, Sigma Prod. No. S-5881 and Sodium Carbonate, Anhydrous, Sigma Prod. No. S-2127.)

H. 0.05% (v/v) Diacetyl Solution (Diacet)  
(Prepare 50 ml in deionized water using Diacetyl, Sigma Prod. No. D-3634.)

I. 100 mM Creatinine Substrate Solution (Creatinine)  
(Prepare 25 ml in deionized water using Creatinine, Free Base, Anhydrous, Sigma Prod. No. C-4255.)

J. 4 mM Creatine Standard Solution (Creat Std)  
(Prepare 10 ml in Reagent C using Creatine, Hydrate, Sigma Prod. No. C-3630.)

K. Creatininase Enzyme Solution  
(Immediately before use, prepare a solution containing 5 – 10 units/ml of Creatininase in cold Reagent C.)

PROCEDURE:

Step 1:

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

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent A (Buffer)</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Reagent I (Creatinine)</td>
<td>0.80</td>
<td>0.80</td>
</tr>
</tbody>
</table>

Mix by swirling and equilibrate to 37°C. Then add:

Reagent K (Enzyme Soln) 0.10

Immediately mix by swirling and incubate at 37°C for exactly 10 minutes. Then add:

Reagent D (Stop Rgt) 2.00 2.00
Reagent K (Enzyme Soln) 0.10
Mix by swirling.
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PROCEDURE:

Step 2:

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

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deionized Water</td>
<td>0.90</td>
<td>0.90</td>
</tr>
<tr>
<td>Reagent E (a-Naphthol)</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>Reagent G (Alk Soln)</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>Reagent H (Diacet)</td>
<td>0.50</td>
<td>0.50</td>
</tr>
</tbody>
</table>

Mix by swirling and then add:

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test Solution</td>
<td>0.10</td>
<td>------</td>
</tr>
<tr>
<td>Blank Solution</td>
<td>------</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Mix by swirling and incubate at 25°C for 1 hour. Then add:

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deionized Water</td>
<td>2.50</td>
<td>2.50</td>
</tr>
</tbody>
</table>

Mix by swirling.

Transfer the Test, Blank, Standards and Standard Blank to suitable cuvettes and read the absorbance at 525nm.

COLOR DEVELOPMENT:

Standard Curve:

Prepare a standard curve by pipetting (in milliliters) the following reagents into suitable containers:

<table>
<thead>
<tr>
<th></th>
<th>Std 1</th>
<th>Std 2</th>
<th>Std 3</th>
<th>Std 4</th>
<th>Std 5</th>
<th>Std Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deionized Water</td>
<td>0.98</td>
<td>0.96</td>
<td>0.94</td>
<td>0.92</td>
<td>0.90</td>
<td>1.00</td>
</tr>
<tr>
<td>Reagent E (a-Naphthol)</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>Reagent G (Alk Soln)</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>Reagent H (Diacet)</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
</tr>
</tbody>
</table>

Mix by swirling and then add:

<table>
<thead>
<tr>
<th></th>
<th>Std Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent J (Creat Std)</td>
<td>0.02 0.04 0.06 0.08 0.10</td>
</tr>
</tbody>
</table>

Mix by swirling and incubate at 25°C for one hour. Then add:

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deionized Water</td>
<td>2.50</td>
<td>2.50</td>
</tr>
</tbody>
</table>
Mix by swirling. Transfer the Standards and Standard Blank to suitable cuvettes and read the absorbance at 525nm.
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CALCULATIONS:

Standard Curve:

\[ r_{A_{525nm}} \text{ Standard} = A_{525nm} \text{ Standard} - A_{525nm} \text{ Standard Blank} \]

Plot the \( r_{A_{525nm}} \text{ Standard} \) vs \( \mu \)moles creatine.

Sample Determination:

\[ r_{A_{525nm}} \text{ Sample} = A_{525nm} \text{ Test} - A_{525nm} \text{ Sample Blank} \]

Determine the \( \mu \)moles of creatinine hydrolyzed using the Standard Curve.

\[
\text{Units/ml enzyme} = \frac{(\mu \text{moles Creatinine hydrolyzed})(3)(df)}{(10)(0.1)(0.1)}
\]

3 = Volume (in milliliters) of stopped reaction  
\( df \) = Dilution factor  
10 = Time of assay (in minutes) as per the Unit Definition  
0.1 = Volume (in milliliters) of stopped reaction used in Colorimetric Determination  
0.1 = Volume (in milliliters) of enzyme used

\[
\text{Units/mg solid} = \frac{\text{units/ml enzyme}}{\text{mg solid/ml enzyme}}
\]

\[
\text{Units/mg protein} = \frac{\text{units/ml enzyme}}{\text{mg protein/ml enzyme}}
\]

UNIT DEFINITION:

One unit will hydrolyze 1.0 \( \mu \)mole of creatinine to creatine per minute at pH 6.5 at 37\(^\circ\)C.

FINAL ASSAY CONCENTRATION:

In a 1.00 ml reaction mix, the final concentrations are 31 mM potassium phosphate, 80 mM creatinine, and 0.5 - 1.0 unit creatininase.
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NOTES:

1. Where Sigma Product or Stock numbers are specified, equivalent reagents may be substituted.

This procedure is for informational purposes. For a current copy of Sigma's quality control procedure contact our Technical Service Department.