Enzymatic Assay of PROTEIN KINASE 3':5'-CYCLIC AMP DEPENDENT Phosphorylating Activity

REACTION:

Casein + $\gamma^{32}$P-ATP $\xrightarrow{\text{Protein Kinase}}$ [$^{32}$P]-Phosphorylated Casein + ADP

CONDITIONS: $T = 30^\circ C$, $pH = 6.5$

METHOD: Radioactive

REAGENTS:

A. 1000 mM Potassium Phosphate Buffer, pH 6.5 at 30°C
(Prepare 100 ml in deionized water using Potassium Phosphate, Monobasic, Anhydrous. Adjust to pH 6.5 at 30°C with 2 M KOH.)

B. 5.0% (w/v) Casein Solution (Casein)
(Use Casein from Bovine Milk, 5% (w/v) Solution.)

C. 500 mM Magnesium Acetate Solution (Mg(OAc)$_2$)
(Prepare 10 ml in deionized water using Magnesium Acetate, Tetrahydrate.)

D. 250 mM Aminophylline Solution (AP)
(Prepare 10 ml in deionized water using Aminophylline, Hydrate.)

E. 330 mM Dithiothreitol Solution (DTT)
(Prepare 10 ml in deionized water using DL-Dithiothreitol. PREPARE FRESH.)

F. 1 mM Adenosine 3':5'-Cyclic Monophosphate Solution (cAMP)
(Prepare 1 ml in deionized water using Adenosine 3':5'-Cyclic Monophosphate, Sodium Salt. PREPARE FRESH.)
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REAGENTS: (continued)

G. 10.0 mM Adenosine 5'-Triphosphate Solution (ATP)  
(Prepare 1 ml in deionized water using Adenosine 5'-Triphosphate, Disodium Salt.  
PREPARE FRESH.)

H. γ-32P-Adenosine 5'-Triphosphate Solution (γ-32P-ATP)  
(Use product with minimum radioactive concentration of 30 curies/m mole and 2 millicuries/ml.)

I. 13.5% (v/v) Trichloroacetic Acid Solution (TCA)  
(Prepare 20 ml in deionized water using Trichloroacetic Acid, 6.1 N Solution, approximately 100% (w/v).)

J. Protein Kinase 3':5'-Cyclic AMP Dependent Enzyme Solution  
(Immediately before use, prepare a solution containing 200 - 400 units/ml of Protein Kinase 3':5'-Cyclic AMP Dependent in cold deionized water.)

K. 6.75% (v/v) Trichloroacetic Acid Solution (Wash)  
(Prepare 20 ml in deionized water using Trichloroacetic Acid, 6.1 N Solution, approximately 100% (w/v).)

L. Methylethyl Cellosolve  
(Prepare 500 ml by adding 250 ml of Ethylene Glycol Monoethyl Ether, to 250 ml of Ethylene Glycol Monomethyl Ether.)

M. Scintillation Cocktail  
(Use Sigma-Fluor Universal LSC Cocktail for Aqueous Samples.)

PROCEDURE:

Prepare a reaction cocktail by pipetting (in milliliters) the following reagents into a suitable container:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deionized Water</td>
<td>1.95</td>
</tr>
<tr>
<td>Reagent A (Buffer)</td>
<td>0.50</td>
</tr>
<tr>
<td>Reagent C (Mg(OAc))</td>
<td>0.25</td>
</tr>
<tr>
<td>Reagent D (AP)</td>
<td>0.10</td>
</tr>
<tr>
<td>Reagent E (DTT)</td>
<td>0.10</td>
</tr>
</tbody>
</table>
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PROCEDURE:  (continued)

Pipette (in milliliters) the following reagents into 2.90 ml of the reaction cocktail.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>F (cAMP)</td>
<td>0.05</td>
</tr>
<tr>
<td>G (ATP)</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Mix by swirling. Transfer 1 ml to a suitable container and add enough Reagent H ($\gamma^{32}$P-ATP) to yield approximately 150,000-200,000 counts/minute (cpm) in 0.05 ml of the solution. Then add 0.50 ml of Reagent B (Casein). This is the Reaction Cocktail.

Pipette 0.05 ml aliquots of Reagent J (Enzyme Solution) into a multiwell disposable titerplate. Place in a 4°C ice bath.

Add 0.05 ml of Reaction Cocktail to each well and mix by air injection. Immediately transfer the titerplate to a 30°C water bath. Incubate at 30°C for 10 minutes. Then add 0.10 ml of Reagent I (TCA) to each well.

Filter the material in the wells through 0.45 µm Millipore HA Type filters. Wash 3 times with Reagent K (Wash).

Transfer the filters to suitable 2 dram scintillation vials containing 2.00 ml of Reagent L (Methylethyl Cellosolve). To each scintillation vial, add 5 ml of Reagent M (Scintillation Cocktail). Count the radioactivity in a suitable scintillation counter.

CALCULATIONS:

The total number of picomoles (pMoles) of ATP in the reaction mixture is calculated as follows:

\[
\frac{(0.05) (0.01) (10^9)}{(3.00) (1.5)} = 1.1111 \times 10^5 \text{ pmole/ml Reaction Cocktail}
\]

Find cpm/pmole by counting 0.05 ml (5555 pmoles) of the Reaction Cocktail.

0.05 = Volume (in milliliters) of ATP used in the Reaction Cocktail
0.01 = Millimolar concentration of ATP (Reagent G)
10^9 = Conversion of millimoles to picomoles
3.00 = Intermediate volume (in milliliters) of Reaction Cocktail
1.5 = Total volume (in milliliters) of Reaction Cocktail
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CALCULATION: (continued)

\[
\text{Units/ml} = \frac{\text{CPM Counted}}{(\text{cpm/pmole}) (10) (0.05)}
\]

CPM counted = Actual count - background on filters
10 = Time of Assay (in minutes) as per the Unit Definition
0.05 = Volume (in milliliters) of enzyme used

UNIT DEFINITION FOR PROTEIN KINASE:

One unit will transfer 1.0 picomole (10^{-12} mole) of phosphate from \( \gamma^{32}\text{P}-\text{ATP} \) to hydrolyzed and partially dephosphorylated casein (C-4765) per minute at pH 6.5 at 30\(^\circ\)C, in the presence of 0.006 mM cyclic-AMP (A6885).

FINAL ASSAY CONCENTRATIONS:

In a 0.10 ml solution, the final assay concentrations are 55 mM potassium phosphate, 14 mM magnesium acetate, 3 mM aminophylline, 4 mM dithiothreitol, 0.006 mM adenosine 3':5'-cyclic monophosphate, 0.055 mM adenosine 5'-triphosphate, 0.83% (w/v) casein, and 10 - 20 units protein kinase 3':5'-cyclic AMP dependent.

REFERENCES:


NOTE:

1. This assay is based on the cited references.

This procedure is for informational purposes.