Enzymatic Assay of PROTEIN KINASE C

PRINCIPLE:

Histone + ?³²P-ATP \( \xrightarrow{\text{Protein Kinase}} \) \[^{32}\text{P} \]-Phosphorylated Histone + ADP

Abbreviations used:
?³²P-ATP = Adenosine 5'-Triphosphate ?³²P-labelled
ADP = Adenosine 5'-Diphosphate

CONDITIONS:  \( T = 30^\circ C, \text{pH} = 7.4 \)

METHOD:  Radiolabelled Stop Reaction

REAGENTS:

A.  1 M Tris HCl Buffer, pH 7.4 at 25°C  
(Prepare 100 ml in deionized water using Trizma Base.  
Adjust to pH 7.4 at 25°C with 1 M HCl.  Store at 4°C.)

B.  4.9 M Magnesium Chloride Solution (MgCl₂)  
(Use Magnesium Chloride, 4.9 M Solution.)

C.  100 mM Ethylene Glycol-bis(ß-Aminoethyl 
Ether)N,N,N',N'-Tetraacetic Acid Solution, pH 7.4 at 
25°C (EGTA)  
(Prepare 50 ml in Reagent A using Ethylene Glycol-bis 
(ß-Aminoethyl Ether)N,N,N',N'-Tetraacetic Acid.  
Adjust to pH 7.4 at 25°C with 1 M NaOH.  Store at 4°C.)

D.  500 mM Calcium Chloride Solution (CaCl₂)  
(Prepare 50 ml in deionized water using Calcium 
Chloride, Dihydrate.  Store at 4°C.)

E.  0.5 mM Adenosine 5'-Triphosphate Solution, pH 7.0 at 
30°C  
(Prepare 10 ml in deionized water using Adenosine 
5'-Triphosphate, Disodium Salt.  
Adjust to pH 7.0 at 30°C with either 1 M HCl or 
1 M NaOH.  Confirm the concentration by using a molar 
extinction coefficient\(^1\) of 15,400 measured at 259 mM.
Store at
−20°C.)
Enzymatic Assay of PROTEIN KINASE C

REAGENTS: (continued)

F. 0.1% (w/v) Triton X-100 Solution (X-100)  
(Prepare 10 ml in deionized water using Triton X-100.)

G. 1 mg/ml Histone Type III-S Solution (Histone)$^2$  
(Prepare 1 ml in deionized water using Histone, Type III-S. The histone used should have a lysine and arginine content greater than or equal to 25.8% and 2.2%, respectively. PREPARE FRESH.)

H. 250 mM Tris HCl Buffer with 100 mM Magnesium Chloride and 10 mM Ethylene Glycol-bis (ß-Aminoethyl Ether)N,N,N',N'-Tetraacetic Acid, pH 7.4 at 30°C  
(Buffer I)  
(Prepare 100 ml using Reagents A, B, and C.)

I. 250 mM Tris HCl Buffer with 100 mM Magnesium Chloride and 5 mM Calcium Chloride, pH 7.4 at 30°C (Buffer II)  
(Prepare 100 ml using Reagents A, B, and D.)

J. 50 mM Tris HCl Buffer with 5 mM $DL$-Dithiothreitol, pH 7.4 at 30°C (Enz Dil)  
(Prepare 25 ml in deionized water using Reagent A and $DL$-Dithiothreitol.)

K. 2 mg/ml Phosphatidylserine Solution (PS)  
(Use $L$-a-Phosphatidyl-$L$-Serine, Chloroform:Methanol (95:5) Solution.)

L. Chloroform Solution  
(Use Chloroform.)

M. 0.5 mg/ml 1,2-Dioleoyl-rac-Glycerol Solution (DAG)  
(Prepare 4 ml in Reagent L using 1,2-Dioleoyl-rac-Glycerol (C18:1,[cis]-9).  
Store under nitrogen in a brown bottle.)

N. Lipid Activator Solution (Lipids)  
(Immediately before use, prepare by pipetting 0.25 ml of Reagent K and 40 µl of Reagent M into a suitable container. Close the container immediately while sealing it under nitrogen. Dry the lipid solution under nitrogen while rotating the container gently. Then add 0.5 ml of Reagent F to the dried lipids. Sonicate in a cold bath sonicator for 1 minute. The solution will become cloudy.)
Enzymatic Assay of PROTEIN KINASE C

REAGENTS: (continued)

O. $^{32}$P-Adenosine 5'-Triphosphate Solution ($^{32}$P-ATP)
(Use $^{32}$P-Adenosine 5'-Triphosphate, 3000 Ci/mmole, 10 mCi/ml.)

P. ATP Reaction Mix (ATP)
(Immediately before use, prepare by adding 1 µl of Reagent O to 100 µl of Reagent E for each of the 9 wells.)

Q. Protein Kinase C Enzyme Solution
(Immediately before use, prepare a solution containing 3.3 - 5.0 units/ml of Protein Kinase C in cold Reagent J.)

R. Scintillation Fluid (Scin Fluid)
(Use Fluor Universal LSC Cocktail for Aqueous Samples.)

S. 10% (v/v) Trichloroacetic Acid Solution (TCA)
(Prepare 1 liter in deionized water using Trichloroacetic Acid, 6.1 N Solution, approximately 100% (w/v).)

T. Nitrocellulose Paper
(Use BA-85 Cellulose Nitrate, Ref. 401-491, Schleicher and Schuell.)

PROCEDURE:

This assay is performed in triplicate in a final volume of 0.05 ml. Each sample is assayed with and without activators. The assay components are added as 0.01 ml aliquots and the last component added is the enzyme solution. The reaction is conducted in a 96 well microtiter plate. A second microtiter plate is used to contain the enzyme solution prior to pipetting with a multichannel pipette.

Plan the assay on a 8 x 12 chart (as indicated) since a 12 row microtiter plate will be used. The first microtiter plate is set up for assaying samples, I-IV, 3 replications each, with (+) and without (-) activators. The second microtiter plate is for the Protein Kinase C samples.

Plate No. 1

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>I</td>
<td>I</td>
<td>I</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>III</td>
<td>III</td>
<td>IV</td>
<td>IV</td>
<td>IV</td>
<td></td>
</tr>
</tbody>
</table>
Enzymatic Assay of PROTEIN KINASE C

PROCEDURE: (continued)

Before conducting the enzyme assay, set up a dot blot blotting apparatus in order that the wells from the microtiter plate can be blotted onto Reagent T (nitrocellulose paper) on top of Whatman 3 mm paper.

Place the microtiter plate on ice. Pipette (in milliliters) the following reagents into 24 of the 96 wells of microtiter Plate No. 1 as previously indicated:

<table>
<thead>
<tr>
<th></th>
<th>Test (-)</th>
<th>Test (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Activator</td>
<td>Activator</td>
</tr>
<tr>
<td>Reagent G (Histone)</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Reagent H (Buffer I)</td>
<td>0.01</td>
<td>-------</td>
</tr>
<tr>
<td>Reagent I (Buffer II)</td>
<td>------</td>
<td>0.01</td>
</tr>
<tr>
<td>Reagent F (X-100)</td>
<td>0.01</td>
<td>-------</td>
</tr>
<tr>
<td>Reagent P (ATP)</td>
<td>0.01</td>
<td>0.01</td>
</tr>
</tbody>
</table>

In a second 96-well microtiter plate, pipette (in milliliter) 0.03 ml, per well, of Reagent Q (Enzyme Solution) as indicated in Plate No. 2.

For controls, pipette 0.03 ml of Reagent J (Enz Dil) into several wells. In the event there are more than 4 samples (including the control) leave one row of space between each row of samples (Plate No. 2). Volumes are to be added with a multichannel pipette from plate No. 2.

Prepare Reagent N (Lipids). Then add:

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent N (Lipids)</td>
<td>------</td>
<td>0.01</td>
</tr>
<tr>
<td>Reagent Q (Enzyme Soln)</td>
<td>0.01</td>
<td>0.01</td>
</tr>
</tbody>
</table>
Enzymatic Assay of PROTEIN KINASE C

PROCEDURE:  (continued)

Incubate at 30°C for exactly 3 minutes; then place on ice. Connect the blot apparatus to a vacuum and transfer 40 µl of the 50 µl contents of the microtiter plate (Plate No. 1) to the parallel place on the blotter. Leave for 5 minutes with vacuum applied.

Disassemble the blot apparatus. Soak the nitrocellulose paper in 200 - 300 ml of Reagent S (TCA). Shake gently for 10 - 15 minutes.

Change to Fresh Reagent S (TCA). Shake for another 10 - 15 minutes. Remove the nitrocellulose paper and place on filter paper to soak up the excess Reagent S (TCA). Cut out the circles (transferred samples) from the nitrocellulose paper and place into scintillation vials containing 1 ml of Reagent R (Scin Fluid). Count the radioactivity in a suitable scintillation counter.

CALCULATIONS:

\[
\text{Units/ml enzyme} = \frac{(\text{cpm})(\text{df})}{(\text{SR})(3)(0.01)} \times \frac{5}{4}
\]

cpm = actual count - background on filter
df = Dilution factor
5/4 = Correction factor for removing 40 µl from the 50 µl reaction mixture
SR = Specific radioactivity (cpm/nmole)
3 = Time (in minutes) of assay as per the Unit Definition
0.01 = Volume (in milliliter) of enzyme used

UNIT DEFINITION:

One unit will transfer 1 nanomole of phosphate from ATP to Histone I (Histone Type III-S) per minute at pH 7.4 at 30°C.

FINAL ASSAY CONCENTRATION:

In a 0.05 ml reaction mix, the final concentrations are 200 µg/ml histone, 60 mM Tris, 20 mM magnesium chloride, 1 mM calcium chloride, 200 µg/ml phosphatidylserine, 8 µg/ml 1,2-dioleoyl-rac-glycerol (C18:1,[cis]-9), 0.02% (w/v) triton X-100, 0.1 mM adenosine 5'-triphosphate, 1 mM DL-dithiothreitol, and 0.03 - 0.05 unit protein.
kinase C.
Enzymatic Assay of PROTEIN KINASE C

REFERENCE:


NOTES:

1. The extinction coefficient is described in Keesey, J. (1987).

2. Histone Type III-S contains the Bradbury H1 fraction as the major fraction.

3. For Protein Kinase C, initially reconstitute the vial in 100 ml of deionized water or 10% (w/v) glycerol if the solution is to be stored frozen.

4. This assay is based on the cited reference.

5. Where our Product or Stock numbers are specified, equivalent reagents may be substituted.

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