

**Enzymatic Assay of ADENYLATE CYCLASE
(EC 4.6.1.1)**

PRINCIPLE:

ATP $\xrightarrow{\text{Adenylate Cyclase}}$ 3',5'-cyclic AMP + Pyrophosphate

Abbreviations used:

ATP = Adenosine 5'-Triphosphate

3',5'-cyclic AMP = 3',5'-cyclic Adenosine Monophosphate

CONDITION: T = 37°C, pH 8.5

METHOD: HPLC Analysis of Products

REAGENTS:

- A. 100 mM Tris HCl Buffer, pH 8.5 at 37°C
(Prepare 100 ml in deionized water using Trizma,
Sigma Prod. No. T-1503. Adjust to pH 8.5 at 37°C with
1 M HCl.)
- B. 20 mM Magnesium Chloride Solution (MgCl₂)
(Prepare 10 ml in deionized water using Magnesium
Chloride, Anhydrous, Sigma Prod. No. M-8266.)
- C. 5.0 mM Sodium Fluoride Solution¹ (NaF)
(Prepare 10 ml in deionized water using Sodium
Fluoride, Sigma Prod. No. S-1504.)
- D. 15 mM Adenosine 5'-Triphosphate Solution, pH 8.5 at
37°C (ATP)
(Prepare 10 ml in deionized water using Adenosine
5'-Triphosphate, Disodium, Sigma Prod. No. A-2383.
Adjust to pH 8.5 at 37°C with 0.1 M NaOH.)
- E. 5.0 mM Phosphocreatine Solution² (PCR)
(Prepare 1 ml in deionized water using
Phosphocreatine, Disodium, Hydrate, Sigma Prod. No. P-
7936.)

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REAGENTS: (continued)

- F. 10 mM Potassium Phosphate and 5 mM Tetrabutylammonium Dihydrogen Phosphate Buffer, pH 5.5 at 25°C (Buffer A) (Immediately before use, prepare 1 L in deionized water using Potassium Phosphate, Monobasic, Anhydrous, Sigma Prod. No. P-0662, and Tetrabutylammonium Dihydrogen Phosphate, Sigma Prod. No. T-1531. Adjust to pH 5.5 at 25°C with 1 M KOH. Degas with nitrogen for 10 minutes.)
- G. 100% (v/v) Acetonitrile (Buffer B) (Use Acetonitrile, Sigma Stock No. 27,071-7. Immediately before use, degas with nitrogen for 10 minutes.)
- H. 1.8 mM Adenosine 5'-Triphosphate Standard Solution (ATP Std) (Prepare 1 ml in deionized water using Adenosine 5'-Triphosphate, Disodium, Sigma Prod. No. A-2383.)
- I. Adenosine 3':5'-Cyclic Monophosphate Standard Solution (3':5'c-AMP) (Immediately before use, prepare 1 ml each of standards in deionized water containing 0.3 mM, 0.6 mM, 0.9 mM, 1.5 mM, Adenosine 3':5'-Cyclic Monophosphate, Free Acid, Sigma Prod. No. A-4137.)
- J. Adenylate Cyclase Enzyme Solution (Immediately before use, prepare a solution containing 50 - 100 units/ml in cold Reagent A.)

PROCEDURE:

Step 1:

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

	<u>Test</u>	<u>Blank</u>
Reagent A (Buffer)	0.50	0.50
Reagent B (MgCl ₂)	0.10	0.10
Reagent C (NaF)	0.10	0.10
Reagent D (ATP)	0.10	0.10
Reagent E (PCR)	0.10	0.10

Mix by swirling and incubate in a water bath at 37°C for 10 minutes. Then add:

Reagent J (Enzyme Solution)	0.02	-----
Deionized Water	0.08	0.10

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PROCEDURE: (continued)

Mix by inversion and incubate at 37°C for exactly 60 minutes. Then place the Test, Blank, and Standard solutions into a boiling water bath for one minute. Allow to cool, then filter through a 0.22 µm filter.

Step 2:

HPLC Analysis

Column: Vydac C18-TBAP, 25 cm x 4.6 mm
 Injection Volume: 20 µl
 Standard: Reagent I (Adenosine 3':5'-Cyclic Monophosphate)
 Flow Rate: 1.5 ml/minute
 Wavelength: 260 nm
 Buffer A: 10 mM Potassium Phosphate and 5 mM Tetrabutylammonium Dihydrogen Phosphate Buffer, pH 5.5 at 25°C
 Buffer B: 100% (v/v) Acetonitrile
 Equilibrate the column with 95% Buffer A and 5% Buffer B for 30 minutes.
 HPLC Program:

<u>Time (Minutes)</u>	<u>0</u>	<u>25</u>	<u>26</u>	<u>35</u>	<u>36</u>	<u>45</u>
Buffer A	95%	70%	0%	0%	95%	95%
Buffer B	5%	30%	100%	100%	5%	5%

Inject 20 µl each of Test, Blank, and Standards. Use at least three different concentrations of the Test. Prepare a standard curve by plotting the peak areas of the standards versus µmoles of 3':5'-AMP. Determine the micromoles of 3':5'-AMP liberated using the standard curve.

CALCULATION:

$$\text{Units/ml enzyme} = \frac{(\mu\text{moles of } 3':5'\text{-AMP})(1)(df)}{(60)(0.001)(0.02)}$$

1 = Volume (in milliliter) of assay
 df = Dilution factor
 60 = Time (in minutes) of assay as per the Unit Definition
 0.001 = Conversion factor from µmoles to nanomoles as per

the Unit Definition
0.02 = Volume (in milliliter) of enzyme used

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CALCULATION: (continued)

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

UNIT DEFINITION:

One unit will form 1.0 nanomole of cyclic AMP from ATP per minute at pH 8.5 at 37°C.

FINAL ASSAY CONCENTRATION:

In a 1.00 ml reaction mix, the final concentrations are 52 mM Tris, 2 mM magnesium chloride, 0.5 mM sodium fluoride, 0.5 mM phosphocreatine, 1.5 mM adenosine 5'-triphosphate, and 1 - 2 units adenylate cyclase.

REFERENCE:

Yang, J.K. and Epstein, W. (1983) *Journal of Biological Chemistry* **258**, 3750 - 3758

NOTES:

1. Sodium Fluoride is included in the assay to inhibit adenosine 5'-triphosphatase.
2. Phosphocreatine is included in the assay to decrease the inhibition from ADP, via phosphocreatine kinase, which is thought to be present in A-0951.
3. This assay is based on the cited reference.
4. 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.