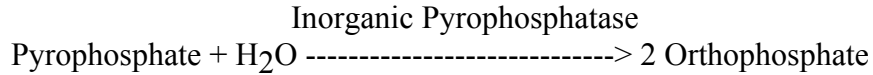


INORGANIC PYROPHOSPHATASE - ENZYMATIC ASSAY



The basis of the colorimetric detection:

The Orthophosphate will react with Ammonium molybdate to form Phosphomolybdic acid. The Phosphomolybdic acid is then reduced by FeSO_4 in a weak acid solution. The blue color produced can be measured at 660nm.

The standard for this reaction is a phosphorous standard solution

11.1 Definitions:

One unit will liberate 1.0 μmole of inorganic orthophosphate per minute at pH 9.0 at 25°C.

11.2 Materials:

Reagent A - 50 mM Tris-HCl buffer, pH 9.0 at 25°C.

Prepare 100 ml in deionized water using Trizma Base. Adjust to pH 9 at 25°C (according to SOP 5-39).

Reagent B - 10 mM Sodium pyrophosphate solution

Dissolve 0.223 g of Pyrophosphate tetrasodium, decahydrate in 50 ml of deionized water.

Reagent C - 10 mM Magnesium chloride solution

Dilute 1 ml of 4.9M Magnesium chloride (Prod. no. 104-20) in 489 ml deionized water. Mix well.

Reagent D - 10% Ammonium molybdate solution in 10N H_2SO_4

1. In the chemical hood prepare 100 ml of 10N H_2SO_4 solution. Dilute 27 ml of H_2SO_4 (34M) in 73 ml of deionized water. Mix well.
Note: You are diluting an acid, be careful.
2. Dissolve 5 g of Ammonium molybdate (Cat no. M0878) in 10N H_2SO_4 , so that the final volume of the solution will be 50 ml. Note: Prepare fresh solution every day.

Reagent E - Tausky-Shorr Reagent

Note: Prepare freshly every day.

1. Add 10 ml 10% Ammonium molybdate in 10N H_2SO_4 , to 80 ml deionized water.
2. Add 5 g of Ferrous sulfate, (Prod. no. F7002). Bring the volume to 100 ml with deionized water. Mix well.

Reagent F - Phosphate standard

Use Phosphate standard solution (Prod. no. P3869). The concentration of the standard is 0.65 $\mu\text{mol/ml}$.

11.3 Equipment and Supplies:

Spectrophotometer

Cuvettes

Dry block suitable for 10-15 ml tubes

10-15 ml tubes

11.4 Method:

Assay conditions:

Temp - 25°C (dry block)

Detection - 660nm

Method: Colorimetric

11.5 Procedure:

A. Enzymatic reaction

1. Dissolve the lyophilized IPP in 2 ml of Reagent A, or use a 1:500 diluted sample before lyophilization.
2. Dilute a sample of the IPP enzyme 200 fold in Reagent A (the dilution factor is 200).
3. Pipette the following reagents into suitable tubes:

Reagents	Blank	Test
Reagent A (TRIS Buffer), ml	4.1	4
Reagent B (Pyrophosphate), ml	1	1
Reagent C (Magnesium chloride), ml	1	1
Enzyme - IPP, ml	-----	0.1

4. Immediately mix and incubate at 25°C for exactly 10 minutes.
Note: During the incubation period prepare the samples for the following color reaction step, i.e. prepare tubes with Reagent E, F and deionized water so that the color reaction will be initiated by the addition of the test mixture/blank mixture/standard.

B Color reaction

1. Pipette the following into suitable tubes:

Reagents	Blank	Test	Standard curve			
			Std1	Std2	Std3	Std Blank
Reagent E, ml (Tausky-Shorr Reagent)	5	5	5	5	5	5.00

Blank reaction, ml	1	---	---	---	---	--
Test reaction, ml	---	1	-----	---	----	---
Reagent F, ml (Phosphate standard)	---	---	0.5 (0.32 μ mol)	1.00 (0.65 μ mol)	1.5 (0.97 μ mol)	---
Deionized water	4	4	4.5	4.00	3.5	5.00

Std = Standard

- Mix and incubate at 25°C for 10 minutes.
- Transfer to 1 ml spectrophotometer cuvettes and record the $A_{660\text{nm}}$ for test, test blank, standards and standard blank.
- IMPORTANT NOTE: TEST AND TEST BLANK COLOUR MAY BE READ ALREADY AFTER 2 minutes OF COLOR REACTION BECAUSE SUBSTRATE IS CHEMICALLY HYDROLYSED AFTER 10 MINUTES BY H₂SO₄!**

11.6 Calculations:

Standard curve:

Prepare a standard curve by plotting the $\Delta A_{660\text{nm}}$ standard vs micromoles of Phosphate
 $\Delta A_{660\text{nm}} \text{Standard} = A_{660\text{nm}} \text{Standard} - A_{660\text{nm}} \text{Standard Blank}$

IPP enzyme activity determination:

Subtract the $A_{660\text{nm}}$ Blank value from the $A_{660\text{nm}}$ Test value
 $\Delta A_{660\text{nm}} \text{ Sample} = A_{660\text{nm}} \text{ Test} - A_{660\text{nm}} \text{ Blank}$

Determine the micromoles of phosphate liberated by the enzyme using the standard curve.

Calculate μmol phosphate release per min (Units) per ml enzyme:

$$\mu\text{mol}/\text{min}/\text{ml enzyme} = \frac{\mu\text{mol Phosphate} \times df}{10 \text{ min} \times 0.1 \text{ ml} \times (1/6.1)} = \frac{\mu\text{mol Phosphate} \times df \times 6.1}{10 \text{ min} \times 0.1 \text{ ml}}$$

where:

6.1 = total volume (in ml) of reaction mix

10 = length of test in minutes

df = Dilution factor

1/6.1 = the part of test mixture used in colorimetric determination

0.1 = Volume (in ml) of enzyme in the enzymatic test

$$\text{Units} / \text{mgP} = \frac{\text{Units} / \text{ml} \text{ enzyme}}{\text{mgP} / \text{ml}}$$

11.7 Unit definition

One unit will liberate 1.0 μ mole of inorganic orthophosphate per minute at pH 9.0 at 25°C.

11.8 Results:

The specific activity should be at least 800 units/mg protein.

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

