Enzymatic Assay of PYRUVATE OXIDASE
(EC 1.2.3.3)

PRINCIPLE:

$$\text{Pyruvate} + O_2 + P_i \xrightarrow[\text{FAD, TPP, } Mg^{2+}]{\text{Pyruvate Oxidase}} \text{Acetylphosphate} + CO_2 + H_2O_2$$

$$2H_2O_2 + \text{4-Aminoantipyrine} + \text{EHSPT} \xrightarrow{\text{POD}} \text{Quinoneimine dye} + 4H_2O$$

Abbreviations used:
P_i = Inorganic Phosphate
FAD = Flavin Adenine Dinucleotide
TPP = Thiamine Pyrophosphate
EHSPT = N-Ethyl-N-(2-Hydroxy-3-Sulfopropyl)-m-Toluidine
POD = Peroxidase

CONDITIONS: T = 37°C, pH 5.7, A_{550nm}, Light path = 1 cm

METHOD: Spectrophotometric Rate Determination

REAGENTS:

A. 150 mM Potassium Phosphate Buffer, pH 5.9 at 37°C
(Prepare 100 ml in deionized water using Potassium Phosphate, Monobasic, Anhydrous, Sigma Prod. No. P-5379. Adjust to pH 5.9 at 37°C with 1 M KOH.)

B. 0.15 mM Flavin Adenine Dinucleotide Solution (FAD)
(Prepare 10 ml in deionized water using Flavin Adenine Dinucleotide, Disodium Salt, Sigma Prod. No. F-6625. **PREPARE FRESH**.)

C. 3 mM Cocarboxylase (Thiamine Pyrophosphate) Solution (TPP)
(Prepare 10 ml in deionized water using Cocarboxylase, Sigma Prod. No. C-8754. **PREPARE FRESH**.)

D. 7.4 mM 4-Aminoantipyrine Solution (4-AAP)
(Prepare 25 ml in deionized water using 4-Aminoantipyrine, Free Base, Sigma Prod. No. A-4382.)

E. Peroxidase Enzyme Solution (POD)
(Immediately before use, prepare a solution containing 50 purpurogallin units/ml in deionized water using Peroxidase, Sigma Prod. No. P-8250.)

REAGENTS: (continued)

F. 150 mM Magnesium Sulfate Solution ($MgSO_4$)
(Prepare 5 ml in deionized water using Magnesium Sulfate, Heptahydrate, Sigma Prod. No. M-1880.)

G. 300 mM Sodium Pyruvate Solution (Pyr)
(Prepare 1 ml in deionized water using Pyruvic Acid, Sodium Salt, Sigma Prod. No. P-2256.)

H. 0.3% (w/v) N-Ethyl-N-(2-Hydroxy-3-Sulfopropyl)-m-Toluidine Solution (EHSPT)
(Prepare 25 ml in deionized water using N-Ethyl-N-(2-Hydroxy-3-Sulfopropyl)-m-Toluidine, Sodium Salt, Sigma Prod. No. E-8631.)

I. 50 mM Potassium Phosphate Buffer, pH 5.7 at 37°C (Enzyme Diluent)
(Prepare 50 ml in deionized water using Potassium Phosphate, Monobasic, Anhydrous, Sigma Prod. No. P-5379. Adjust to pH 5.7 at 37°C with 1 M KOH.)

J. 15 mM Ethylenediaminetetraacetic Acid Solution (EDTA)
(Prepare 25 ml in deionized water using Ethylenediaminetetraacetic Acid, Disodium Salt, Dihydrate, Sigma Stock No. ED2SS.)

K. Pyruvate Oxidase Enzyme Solution
(Immediately before use, prepare a solution containing 0.2 - 0.4 unit/ml of Pyruvate Oxidase in cold Reagent I.)

PROCEDURE:

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

Reagent A (Buffer)	10.00
Reagent D (4-AAP)	2.00
Reagent H (EHSPT)	2.00
Reagent C (TPP)	2.00
Reagent B (FAD)	2.00
Reagent J (EDTA)	2.00
Reagent F ($MgSO_4$)	2.00
Reagent E (POD)	3.00

Mix by swirling.

Enzymatic Assay of PYRUVATE OXIDASE
(EC 1.2.3.3)

PROCEDURE: (continued)

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

	Test	Blank
Reaction Cocktail	2.50	2.50
Reagent G (Pyr)	0.50	0.50

Mix by inversion and equilibrate to 37°C. Monitor the A_{550nm} until constant, using a suitably thermostatted spectrophotometer. Then add:

Reagent I (Enzyme Diluent)	------	0.10
Reagent K (Enzyme Solution)	0.10	------

Immediately mix by inversion and record the increase in A_{550nm} for approximately 5 minutes. Obtain the ΔA_{550nm}/min using the maximum linear rate for both the Test and Blank.

CALCULATIONS:

$$\text{Units/ml enzyme} = \frac{(\Delta A_{550nm}/\text{min Test} - \Delta A_{550nm}/\text{min Blank})(3.1)(df)}{(36.88)(0.1)(0.5)}$$

3.1 = Volume (in milliliters) of assay
df = Dilution factor
36.88 = Millimolar extinction coefficient of quinoneimine dye
under the assay conditions
0.1 = Volume (in milliliter) of enzyme used
0.5 = Factor based on the equation that one mole of H_2O_2 produces half a mole of quinoneimine dye

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

UNIT DEFINITION:

One unit will produce 1.0 µmole of H_2O_2 per minute at pH 5.7 at 37°C during the conversion of pyruvate and phosphate to acetylphosphate and CO_2.

Enzymatic Assay of PYRUVATE OXIDASE (EC 1.2.3.3)

FINAL ASSAY CONCENTRATIONS:

In a 3.10 ml reaction mix, the final concentrations are 50 mM potassium phosphate, 0.48 mM 4-aminoantipyrene, 0.02% (w/v) N-ethyl-N-(2-hydroxy-3-sulfopropyl)-m-toluidine, 0.2 mM cocarboxylase, 0.0097 mM flavin adenine dinucleotide, 0.97 mM ethylenediaminetetraacetic acid, 9.7 mM magnesium sulfate, 48 mM sodium pyruvate, 15 units peroxidase, and
0.02 - 0.04 unit pyruvate oxidase.

REFERENCES:

Sedewitz, B., Schleifer, K.H. and Götz, F. (1984) *Journal of Bacteriology* **160**, 273-278.

NOTES:

1. This assay is based on the cited reference.

2. Peroxidase Unit Definition: One unit will form 1.0 mg purpurogallin from pyrogallol in 20 seconds at pH 6.0 at 25°C.

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