Enzymatic Assay of D-AMINO ACID OXIDASE APOENZYME (Reactivation Assay)[1]

PRINCIPLE:

$$\text{D-Alanine} + O_2 + H_2O \xrightarrow[\text{FAD}]{\text{D-Amino Acid Oxidase Apoenzyme}} \text{Pyruvate} + NH_3 + H_2O_2$$

$$2\ H_2O_2 \xrightarrow{\text{Catalase}} 2\ H_2O + O_2$$

$$\text{Pyruvate} + \text{ß-NADH} \xrightarrow{\text{LDH}} \text{L-Lactic Acid} + \text{ß-NAD}$$

Abbreviations used:
FAD = Flavin Adenine Dinucleotide
ß-NADH = ß-Nicotinamide Adenine Dinucleotide, Reduced Form
ß-NAD = ß-Nicotinamide Adenine, Dinucleotide, Oxidized Form

CONDITIONS: T = 25°C, pH = 8.3, A_{340nm}, Light path = 1 cm

METHOD: Continuous Spectrophotometric Rate Determination

REAGENTS:

A. 200 mM Tris HCl Buffer, pH 8.3 at 25°C
(Prepare 100 ml in deionized water using Trizma Base, Sigma Prod. No. T-1503. Adjust to pH 8.3 at 25°C with 1 M HCl.)

B. 224 mM D-Alanine Solution (D-Ala)
(Prepare 5 ml in deionized water using D-Alanine, Sigma Prod. No. A-7377.)

C. 6.4 mM ß-Nicotinamide Adenine Dinucleotide, Reduced Form (ß-NADH)
(Dissolve the contents of one 5 mg vial of ß-Nicotinamide Adenine Dinucleotide, Reduced Form, Disodium Salt,
Sigma Stock No. 340-105, in the appropriate volume of Reagent A. **PREPARE FRESH**.)

D. Catalase Enzyme Solution (Catalase)
(Immediately before use, prepare a solution containing 600 units/ml of Catalase, Sigma Stock No. C-100, in cold deionized water.

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REAGENTS: (continued)

E. L-Lactic Dehydrogenase Enzyme Solution (LDH)
(Immediately before use, prepare a solution containing 400 units/ml of L-Lactic Dehydrogenase, Sigma Prod. No. L-2500, in cold deionized water.)

F. D-Amino Acid Oxidase Apoenzyme (D-AAO)
(Immediately before use, prepare a solution containing 1 mg/ml of D-Amino Acid Oxidase Apoenzyme in cold deionized water.)

G. 50 mM Pyrophosphate Buffer, pH 8.5 at 25°C (PPi)
(Prepare 5 ml in deionized water using Pyrophosphate, Tetrasodium, Decahydrate, Sigma Prod. No. P-9146. Adjust to pH 8.5 at 25°C with 1 M HCl.)

H. 60 mM Flavin Adenine Dinucleotide Solution (FAD)
(Prepare 1 ml in Reagent G using Flavin Adenine Dinucleotide, Disodium Salt, Sigma Prod. No. F-6625.)

PROCEDURE:

Reactivation of D-Amino Acid Oxidase Apoenzyme: Combine 1 ml of Reagent F (D-AAO) with 0.1 ml of Reagent H (FAD). Incubate at 25°C for 30 - 45 minutes.

Saturate Reagent A (Buffer) with O_2 by bubbling oxygen gas through Reagent A (Buffer) for 5 minutes immediately before use.

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

	Test	Blank
Reagent A (O_2 Saturated Buffer)	2.25	2.25
Reagent B (D-Ala)	0.50	0.50
Reagent C (ß-NADH)	0.05	0.05
Reagent D (Catalase)	0.05	0.05
Reagent E (LDH)	0.05	0.05

Mix by inversion and equilibrate to 25°C. Monitor the A_{340nm} until constant, using a suitably thermostatted spectrophotometer. Then add:

Reactivated D-Amino Oxidase Apoenzyme	0.10	------
Deionized Water	------	0.10

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PROCEDURE: (continued)

Immediately mix by inversion and record the decrease in A_{340nm} for approximately 5 minutes. Obtain the $r\ A_{340nm}$/minute using the maximum linear rate for both the Test and Blank.

CALCULATIONS:

$$\text{Units/ml enzyme} = \frac{(r\ A_{340nm}/\text{min Test} - r\ A_{340nm}/\text{min Blank})(3)(df)}{(6.22)(0.1)}$$

3 = Total volume (in milliliters) of the assay
df = Dilution factor
6.22 = Millimolar extinction coefficient of ß-NADH at 340 nm
0.1 = Volume (in milliliter) of enzyme used in assay

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

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

UNIT DEFINITION:

One unit will oxidatively deaminate 1.0 µmole of D-alanine to pyruvate per minute at pH 8.3 at 25°C, in the presence of catalase.

FINAL ASSAY CONCENTRATIONS:

In a 3.00 ml reaction mix, the final concentrations are 153 mM Tris, 37 mM D-alanine, 0.11 mM ß-nicotinamide adenine dinucleotide, reduced form, 30 units catalase, 20 units L-lactic dehydrogenase, 0.2 mM flavin adenine dinucleotide, 0.2 mM pyrophosphate, and 0.1 mg D-amino acid oxidase apoenzyme.

REFERENCE:

Bergmeyer, H.U., Gawehn, K., and Grassl, M. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H.U., ed.) 2nd ed., Volume I, 431-432, Academic Press, New York, NY

Massey, V. and Curti, B. (1966) *Journal of Biological Chemistry* **241**, 3417-3423

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NOTES:

1. This assay is used to measure the D-Amino Acid Oxidase activity of D-Amino Acid Oxidase Apoenzyme, after it has been reactivated by incubating it with flavin adenine dinucleotide.

2. Catalase Unit Definition: One unit will decompose 1.0 µmole of H_2O_2 per minute at pH 7.0 at 25°C, while the H_2O_2 concentration falls from 10.3 to 9.2 mM. The rate of disappearance of H_2O_2 is followed by observing the rate of decrease in absorbance at 240 nm.

3. L-Lactic Dehydrogenase Unit Definition: One unit will reduce 1.0 µmole of pyruvate to L-lactate per minute at pH 7.5 at 37°C.

4. This assay is based on the cited reference.

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