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

**PRINCIPLE:**

\[
\text{D-Alanine} + O_2 + H_2O \xrightarrow{\text{D-Amino Acid Oxidase Apoenzyme}} \text{Pyruvate} + \text{NH}_3 + H_2O_2
\]

\[
2 \text{H}_2\text{O}_2 \xrightarrow{\text{Catalase}} 2 \text{H}_2\text{O} + O_2
\]

\[
\text{Pyruvate} + \beta\text{-NADH} \xrightarrow{\text{LDH}} \text{L-Lactic Acid} + \beta\text{-NAD}
\]

Abbreviations used:
- FAD = Flavin Adenine Dinucleotide
- \(\beta\)-NADH = \(\beta\)-Nicotinamide Adenine Dinucleotide, Reduced Form
- \(\beta\)-NAD = \(\beta\)-Nicotinamide Adenine, Dinucleotide, Oxidized Form

**CONDITIONS:** \(T = 25^\circ\text{C}, \text{pH} = 8.3, A_{340nm}, \text{Light path} = 1 \text{ cm}\)

**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 \(\beta\)-Nicotinamide Adenine Dinucleotide, Reduced Form (\(\beta\)-NADH)
   (Dissolve the contents of one 5 mg vial of \(\beta\)-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 O₂ by bubbling oxygen gas
through Reagent A (Buffer) for 5 minutes immediately
before use.

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

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Test</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent A (O₂ Saturated Buffer)</td>
<td>2.25</td>
<td>2.25</td>
</tr>
<tr>
<td>Reagent B (D-Ala)</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>Reagent C (ß-NADH)</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Reagent D (Catalase)</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Reagent E (LDH)</td>
<td>0.05</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Mix by inversion and equilibrate to 25°C. Monitor the
A₃₄₀nm until constant, using a suitably thermostatted
spectrophotometer. Then add:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Test</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reactivated D-Amino Oxidase Apoenzyme</td>
<td>0.10</td>
<td>------</td>
</tr>
<tr>
<td>Deionized Water</td>
<td>------</td>
<td>0.10</td>
</tr>
</tbody>
</table>
Enzymatic Assay of D-AMINO ACID OXIDASE APOENZYME
(Reactivation Assay)\(^1\)

**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:**

\[
\text{Units/ml enzyme} = \frac{\left( r \frac{\text{A}_{340nm}}{\text{min Test}} - r \frac{\text{A}_{340nm}}{\text{min Blank}} \right) (3)(\text{df})}{(6.22)(0.1)}
\]

3 = Total volume (in milliliters) of the assay
\(\text{df} = \) Dilution factor
6.22 = Millimolar extinction coefficient of \(\beta\)-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 \(\mu\)mole of \(D\)-alanine
to pyruvate per minute at pH 8.3 at 25\(^\circ\)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 \(\beta\)-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:**

Methods of Enzymatic Analysis (Bergmeyer, H.U., ed.) 2nd

Massey, V. and Curti, B. (1966) *Journal of Biological
Chemistry* 241, 3417-3423
Enzymatic Assay of $\alpha$-AMINO ACID OXIDASE APOENZYME
(Reactivation Assay)$^1$

NOTES:

1. This assay is used to measure the $\alpha$-Amino Acid Oxidase activity of $\alpha$-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 $\mu$ mole 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. $\iota$-Lactic Dehydrogenase Unit Definition: One unit will reduce 1.0 $\mu$ mole of pyruvate to $\iota$-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.

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