

**Enzymatic Assay of ALLANTOINASE
(EC 3.5.2.5)**

PRINCIPLE:

Allantoin + H₂O $\xrightarrow{\text{Allantoinase}}$ Allantoate

Allantoate + K₃Fe(CN)₆ + Phenylhydrazine $\xrightarrow{\text{HCl}}$ Glyoxylate Colored Product

CONDITIONS: T = 25°C, pH = 7.0, A_{540nm}, Light path = 1 cm

METHOD: Colorimetric

REAGENTS:

- A. 100 mM Tris HCl Buffer, pH 7.0 at 25°C
(Prepare 100 ml in deionized water using Trizma Base, Sigma Prod. No. T-1503. Adjust to pH 7.0 at 25°C with 1 M HCl.)
- B. 33 mM Allantoin Solution, pH 7.0 at 25°C (Allantoin)
(Prepare 50 ml in Reagent A using Allantoin, Sigma Prod. No. A-7878. Adjust to pH 7.0 at 25°C with either 1 M HCl or 1 M NaOH.)
- C. 69 mM Phenylhydrazine HCl Solution (PH)
(Prepare 10 ml in deionized water using Phenylhydrazine Hydrochloride, Sigma Prod. No. P-6926.)
- D. 25% (v/v) Hydrochloric Acid Solution (HCl)
(Prepare 25 ml in deionized water using Hydrochloric Acid, Sigma Prod. No. H-7020.)
- E. 152 mM Potassium Ferricyanide Solution (K₃Fe(CN)₆)
(Prepare 10 ml in deionized water using Potassium Ferricyanide, Sigma Prod. No. P-8131.)
- F. 0.1 mM Glyoxylic Acid Standard Solution (Std)
(Prepare 5 ml in deionized water using Glyoxylic Acid, Sodium Salt, Monohydrate, Sigma Prod. No. G-4502.)
- G. Allantoinase Enzyme Solution
(Immediately before use, prepare a solution containing

0.05 - 0.20 unit/ml of Allantoinase in cold Reagent
A.)

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PROCEDURE:

Step 1:

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

	<u>Test</u>	<u>Blank</u>
Reagent B (Allantoin)	4.00	4.00

Equilibrate to 25°C. Then add:

Reagent G (Enzyme Solution)	1.00	-----
Reagent A (Buffer)	-----	1.00

Immediately mix by swirling and incubate at 25°C for exactly 5 minutes. Remove aliquots from the Test and Blank at 5, 10, and 15 minutes.

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

	<u>Test¹</u>	<u>Test Blank</u>	<u>Std 1</u>	<u>Std 2</u>	<u>Std 3</u>	<u>Std 4</u>	<u>Std Blank</u>
Reagent D (HCl)	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Reagent C (PH)	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Test Mixture	1.00	----	----	----	----	----	----
Test Blank Mixture	----	1.00	----	----	----	----	----
Reagent F (Std)	----	----	0.20	0.40	0.60	0.80	----
Deionzed Water	----	----	0.80	0.60	0.40	0.20	1.00

Mix by swirling and boil for 2 minutes. Place on ice. Then add:

Reagent D (HCl)	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Reagent E ($K_3Fe(CN)_6$)	0.05	0.05	0.05	0.05	0.05	0.05	0.05

0.05

Allow to incubate on ice for 20 minutes. Then add:

Deionzied Water	2.00	2.00	2.00	2.00	2.00	2.00	2.00
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Mix by swirling and transfer the solutions to suitable cuvettes. Record the A_{540nm} for the Tests, Test Blank, Standards, and Standard Blank using a suitable spectrophotometer.

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CALCULATIONS:

Standard Curve:

$$r A_{540\text{nm}} \text{ Standard} = A_{540\text{nm}} \text{ Standard} - A_{540\text{nm}} \text{ Standard Blank}$$

Prepare a standard curve by plotting $r A_{540\text{nm}}$ Standard vs μmoles of glyoxylic acid.

Sample Determination:

$$r A_{540\text{nm}} \text{ Test} = A_{540\text{nm}} \text{ Test} - A_{540\text{nm}} \text{ Test Blank.}$$

Determine the μmoles of glyoxylic acid liberated using the standard curve.

$$\text{Units/ml enzyme} = \frac{(\mu\text{moles of glyoxylic acid released})(5)(\text{df})}{(1) (T)}$$

5 = Total volume (in milliliters) of reaction mix

df = Dilution factor

1 = Volume (in milliliter) of enzyme used

T = Time (in minutes) of assay as per the Unit Definition

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

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

$$\text{Units/g protein} = \text{units/mg protein} \times 1000$$

UNIT DEFINITION:

One unit will hydrolyze 1.0 μmole of allantoin to allantoate (measured as glyoxylate) per minute at pH 7.0 at 25°C.

FINAL ASSAY CONCENTRATIONS:

In a 5.00 ml reaction mix, the final concentrations are 27 mM allantoin, 100 mM Tris, and 0.05 - 0.20 unit allantoinase.

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REFERENCE:

Hong, Y.-N. and Schopfer, P. (1981) *Planta* **152**, 325-335

Dawson, R.M.C., Elliott, D.C., Elliott, W.H. and Jones, K.M. (1990) in *Data for Biochemical Research*, 3rd ed., 4-5, Oxford University Press, New York, NY

NOTES:

1. Aliquots from the Test and Blank are to be removed at 5, 10, and 15 minutes time points. A standard curve of glyoxylic acid must be prepared for each time point, as the color intensity may vary with time.
2. This assay is based on the cited references.
3. 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.