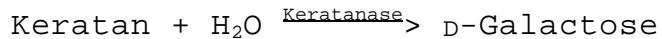


**Enzymatic Assay of KERATANASE
(EC 3.2.1.103)**

PRINCIPLE:



CONDITIONS: T = 37°C, pH = 7.4, A_{690nm}, Light path = 1 cm

METHOD: Colorimetric

REAGENTS:

- A. 50 mM Tris HCl Buffer, pH 7.4 at 37°C
(Prepare 100 ml in deionized water using Trizma Base, Sigma Prod. No. T-1503. Adjust to pH 7.4 at 37°C with 1 M HCl.)
- B. 0.06% (w/v) Keratan Sulfate Solution (Ker)
(Prepare 5 ml in cold Reagent A using Keratan Sulfate, Sigma Prod. No. K-3001.)
- C. 0.05% (w/v) Ferricyanide Solution (K₃Fe(CN)₆)
(Prepare 10 ml in deionized water using Potassium Ferricyanide, Sigma Prod. No. P-8131. **STORE IN AN AMBER BOTTLE-LIGHT SENSITIVE.**)
- D. 50 mM Sodium Carbonate with 10 mM Potassium Cyanide Solution (Na₂CO₃-KCN)
(Prepare 1 liter in deionized water using Sodium Carbonate, Anhydrous, Sigma Prod. No. S-2127, and Potassium Cyanide, Aldrich Stock No. 20,781-0.)
- E. 3.1 mM Ferric Ammonium Sulfate with 0.1% (w/v) SDS and 25 mM Sulfuric Acid Solution (Fe(III))
(Prepare 1 liter in 25 mM Sulfuric Acid using Ferric Ammonium Sulfate, Sigma Prod. No. F-3629, Lauryl Sulfate, Sodium Salt (SDS), Sigma Prod. No. L-5750, and Sulfuric Acid, Sigma Prod. No. S-1526.)
- F. 0.1 mM (w/v) Galactose Standard Solution (Gal)
(Prepare 10 ml in deionized water using D(+)Galactose, Sigma Prod. No. G-0750.)

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REAGENTS: (continued)

G. Keratanase Enzyme Solution
(Immediately before use, prepare a solution containing
5 units/ml of Keratanase in cold Reagent A.)

PROCEDURE:

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

	<u>Test</u>	<u>Blank</u>
Reagent B (Ker)	0.18	0.20

Equilibrate to 37°C. Then add:

Reagent G (Enzyme Solution)	0.02	-----
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Mix by swirling and incubate at 37°C for exactly 15
minutes. After 15 minutes, place the Test and Blank vials
into a boiling water bath for 2 minutes. Remove the vials
and allow to cool to room temperature. Then add:

Deionized Water	0.80	0.80
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Mix by inversion. Then add:

Reagent C ($K_3Fe(CN)_6$)	1.00	1.00
Reagent D (Na_2CO_3-KCN)	1.00	1.00

Mix by swirling and place the vials into a boiling water
bath for 15 minutes. Allow to cool to room temperature.
Then add:

Reagent E (Fe(III))	5.00	5.00
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Mix by swirling and let stand at 25°C for 15 minutes.
Transfer to suitable cuvettes and record the A_{690nm} for both
the Test and Blank using a suitable spectrophotometer.

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PROCEDURE: (continued)

Standard Curve:

A standard curve is made by pipetting (in milliliters) the following reagents into suitable containers:

	Std 1	Std 2	Std 3	Std 4	Std 5	Std Blank
Reagent F (Gal)	0.10	0.30	0.50	0.70	1.00	-----
Deionized Water	0.90	0.70	0.50	0.30	-----	1.00
Reagent C (K ₃ Fe(CN) ₆)	1.00	1.00	1.00	1.00	1.00	1.00
Reagent D (Na ₂ CO ₃ -KCN)	1.00	1.00	1.00	1.00	1.00	1.00

Place the containers into a boiling water bath for 15 minutes. Allow to cool to room temperature. Then add:

Reagent E (Fe(III))	5.00	5.00	5.00	5.00	5.00	5.00
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Mix by swirling and let stand at 25°C for 15 minutes. Transfer to suitable cuvettes and record the A_{690nm} for the Standards and Blank.

CALCULATIONS:

Standard Curve:

$$\Delta A_{690nm} \text{ Standard} = A_{690nm} \text{ Std} - A_{690nm} \text{ Std Blank}$$

Plot the ΔA_{690nm} Standards vs μmoles of reducing sugar.

Sample Determination:

$$\Delta A_{690nm} \text{ Sample} = A_{690nm} \text{ Test} - A_{690nm} \text{ Test Blank}$$

Determine the μmoles of reducing sugar liberated using the Standard Curve.

$$\text{Units/vial enzyme} = \frac{(\mu\text{moles of reducing Sugar liberated})(4)(df)}{(0.02)}$$

4 = Conversion factor for minutes to hours as per the Unit Definition

0.02 = Volume (in milliliter) of enzyme used in assay

df = Dilution factor

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UNIT DEFINITION:

One unit will liberate 1.0 μ mole of reducing sugar (measured as galactose) from keratan sulfate per hour at pH 7.4 at 37°C.

FINAL ASSAY CONCENTRATIONS:

In a 0.20 ml reaction mix, the final concentrations are 50 mM Tris, 0.05% (w/v) keratan and 0.1 unit keratanase.

REFERENCE:

Park, J.T. and Johnson, M.J. (1949) *Journal of Biological Chemistry* **181**, 149-151

NOTES:

1. All product and stock numbers, unless otherwise indicated, are Sigma product and stock numbers.

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