

Enzymatic Assay of HYALURONIDASE¹ (EC 3.2.1.35)

PRINCIPLE:

Hyaluronic Acid $\xrightarrow{\text{Hyaluronidase}}$ Depolymerization Products + Hyaluronic Acid

CONDITIONS: T = 37°C, pH = 5.90, A_{600nm}, Light Path = 1 cm

METHOD: Turbidimetric

REAGENTS:

- A. 300 mM Sodium Phosphate pH 5.35 at 37°C.
(Prepare 100 ml in deionized water using Sodium Phosphate, Monobasic, Anhydrous, Sigma Prod. No. S-0751. Adjust the pH to 5.35 at 37°C using 1 M NaOH.)
- B. 0.30% (w/v) Hyaluronic Acid Solution
(Prepare 5 - 10 ml of a 3 mg/ml solution using Hyaluronic Acid, Sigma Prod. No. H-7630, in Reagent A. Heat until just before boiling and stir for approximately 20 minutes to dissolve.)
- C. 0.03% (w/v) Hyaluronic Acid Solution (Substrate Solution)
(Prepare 10 ml in Reagent A using Reagent B. Prewarm to 37°C immediately before use.)
- D. 20 mM Sodium Phosphate pH 7.0 at 37°C with
77 mM Sodium Chloride and 0.01% (w/v) Bovine Serum Albumin (Enz Dil)
(Prepare 150 ml in deionized water using Sodium Phosphate, Monobasic, Anhydrous, Sigma Prod. No. S-0751, Sodium Chloride, Sigma Prod. No. S-9625, and Albumin, Bovine, Sigma Prod. No. A-6003. Adjust to pH 7.0 using 1 M NaOH.)
- E. 24 mM Sodium Acetate with 79 mM Acetic Acid and
0.1% (w/v) Bovine Serum Albumin pH 3.75 at 37°C containing 1:1 HCl:H₂O (Acid Albumin Soln)
(Prepare 100 ml in deionized water using Sodium Acetate, Trihydrate, Sigma Prod. No. S-8625, Glacial Acetic Acid, Sigma Prod. No. A-0808, and Albumin, Bovine, Sigma Prod. No. A-6003. Adjust to pH 3.75 at 37°C using 5 N HCl.)

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REAGENTS: (continued)

- F. Hyaluronidase Enzyme Solution
(Immediately before use prepare a solution containing 3 - 5 units per ml in cold Reagent D.)
- G. National Formulary (NF) Standard Hyaluronidase Solution (NF Std)
(Immediately before use prepare a solution containing 10 units NF Standard Hyaluronidase per ml in cold Reagent D.)

PROCEDURE:

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

| | Test 1 | Test 2 | Test 3 | Blk | Std 1 | Std 2 | Std 3 | Std 4 | Std 5 | Std 6 |
|----------------------------|--------|--------|--------|------|-------|-------|-------|-------|-------|-------|
| Reagent F (Enzyme) | 1.00 | 0.75 | 0.50 | --- | --- | --- | --- | --- | --- | --- |
| Reagent G (NF Std) | --- | --- | --- | --- | 0.10 | 0.20 | 0.30 | 0.40 | 0.50 | 0.60 |
| Reagent D (Enz Dil) | --- | 0.25 | 0.50 | 1.00 | 0.90 | 0.80 | 0.70 | 0.60 | 0.50 | 0.40 |

Incubate at 37°C for 10 minutes. Then add at time zero:

| | | | | | | | | | | |
|------------------------------|------|------|------|------|------|------|------|------|------|------|
| Reagent C (Substrate) | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
|------------------------------|------|------|------|------|------|------|------|------|------|------|

Mix by inversion and incubate at 37°C for exactly 45 minutes. Pipette 0.50 ml of the above reaction mixture into a cuvette containing 2.5 ml of Reagent E (Acid Albumin Solution). Immediately mix by inversion. Allow the cuvettes to stand at room temperature for exactly 10 minutes. Read the absorbance (A_{600nm}) of the standard curve versus the blank on the spectrophotometer and analyze it using quadratic regression. Read the absorbance (A_{600nm}) of the tests versus the same blank. Set up the spectrophotometer to determine the Hyaluronidase units for the tests versus the standard curve.

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CALCULATIONS:

$$\Delta A_{600\text{nm}} \text{ Sample} = A_{600\text{nm}} \text{ Test} - A_{600\text{nm}} \text{ Blank}$$

$$\Delta A_{600\text{nm}} \text{ Standard} = A_{600\text{nm}} \text{ Standard} - A_{600\text{nm}} \text{ Blank}$$

$$\text{Units/ml enzyme} = \frac{(\text{Hyaluronidase units from Std Curve})(\text{df})}{(\text{Enzyme aliquot})}$$

df = Dilution factor

Enzyme aliquot = Volume (in milliliters) of enzyme used in assay

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

UNIT DEFINITION:

One unit is equivalent to one NF unit as determined by the method described in USP XXII-NF XVII combined edition, p. 644 (1990).

FINAL ASSAY CONCENTRATIONS:

In a 2.00 ml reaction mix, the final concentrations are 160 mM sodium phosphate, 39 mM sodium chloride, 0.005% (w/v) bovine serum albumin, 0.015% (w/v) hyaluronic acid, and 1.5 - 5 units hyaluronidase.

REFERENCES:

Dorfman, A. (1955) *Methods in Enzymology*, Volume I, 166-173

USP XXII-NF XVII (1990) 644-645, United States Pharmacopeia Convention, Inc., Rockville, MD

NOTES:

1. This assay is based on Dorfman, A. (1955).
2. 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.