

Enzymatic Assay of HIRUDIN¹

PRINCIPLE:

Hirudin + Thrombin \longrightarrow Hirudin/Thrombin complex + Thrombin (excess)

Plasma (Fibrinogen) $\xrightarrow{\text{Thrombin (excess)}}$ Clot (Fibrin)

CONDITIONS: T = 37°C, pH = 7.35

METHOD: Fibrometer

- A. 36 mM Diethyl Barbiturate and 36 mM Sodium Acetate, 0.85% (w/v) Sodium Chloride Solution, pH 7.35 at 37°C (Prepare 100 ml using Sodium Acetate, Trihydrate, Sigma Prod. No. S-8625, Barbitol, Sodium Salt, Sigma Prod. No. B-0500, and Sodium Chloride, Sigma Prod. No. S-9625. Adjust the pH to 7.35 at 37°C with 1 M HCl.²)
- B. 0.85% (w/v) Sodium Chloride Solution (Prepare 100 ml in deionized water using Sodium Chloride, Sigma Prod. No. S-9625.)
- C. 7.2 mM Diethyl Barbiturate, 7.2 mM Sodium Acetate, 5.1 mM Sodium Citrate, and 0.68% (w/v) Sodium Chloride (Prepare 100 ml by combining 20 ml of Reagent A, 60 ml of Reagent B, and the appropriate amount of Citric Acid, Trisodium Salt, Dihydrate, Sigma Prod. No. C-7254. Adjust the pH to 7.35 at 37°C with 1 N HCl or 1 M NaOH.³)
- D. 7.2 mM Diethyl Barbiturate, 7.2 mM Sodium Acetate, 5.1 mM Sodium Citrate, 0.68% (w/v) Sodium Chloride, 1.0% (w/v) Bovine Serum Albumin and 0.5% (w/v) Polyethylene Glycol (Enz Dil) (Prepare 20 ml in Reagent C using Albumin, Bovine, Sigma Prod. No. A-4503, and Polyethylene Glycol, Sigma Prod. No. P-2139.)
- E. 50% (w/v) Normal Human Plasma (Immediately before use, reconstitute 2 vials of Human Plasma, lyophilized with 2 ml each of deionized water. Let stand 5 minutes. Then add 2 ml of Reagent B (NaCl) to each. Combine vials and keep at room temperature.⁴)

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REAGENTS: (continued)

- F. Thrombin Enzyme Solution (Thrombin)⁵
(Immediately before use, reconstitute a vial of Thrombin, Sigma Prod. No. T-8885, with 1 ml of deionized water. Assay for units/ml of Thrombin using the Thrombin assay. After determining units/ml of Thrombin of this solution, further dilute to 9.8 - 10.8 units/ml of Thrombin using Reagent D. Assay this solution again for units/ml of Thrombin and use this volume as the working Thrombin Solution.)
- G. NIH Thrombin Standard Solution (Std)
(Use Thrombin Reference Standard (Lot J) which has been diluted in Reagent D. Note: Current Standard Curve has been made with N.I.H. Thrombin standard diluted in glass test tubes. Next standard curve will use Plastic test tubes.)
- H. Hirudin Solution (Hirudin)
(Immediately before use, prepare a solution containing approximately 10 units/ml using Reagent D at room temperature.)⁶

PROCEDURE:

Pipette (in milliliters) the following reagents into a test tube:

	<u>Test</u>
Reagent F (Thrombin)	0.20
Reagent H (Hirudin)	0.10

Incubate at 37°C in a water bath for minimum of 1 minute. After 1 minute, but before 3 minutes, remove 0.1 ml of the assay mix and assay it for excess thrombin activity using the thrombin assay. Repeat the assay with another 0.1 ml aliquot. The excess thrombin is stable over the 1 - 3 minute time period used for the assay. Using the time it takes to stop the fibrometer (the time should be in the 15 - 25 second range and give a % inhibition of thrombin of between 45 and 55%) and the thrombin standard curve, determine the thrombin concentration in units/ml.

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CALCULATIONS: (continued)

$$\text{Units/ml H} = \frac{[(T \text{ units/assay}) - (\text{Excess T units})]}{(0.1)(\text{ml Hirudin/ml assay mix}) \times \text{df}}$$

$$\text{Units/vial H} = \frac{\text{Units/ml H}}{\text{vial H} / \text{mL}}$$

$$\% \text{ Inhibition}^7 = \frac{[(T \text{ units/assay}) - (\text{Excess T units})] \times 100}{(T \text{ units/assay})}$$

$$\begin{aligned} T \text{ Units/assay} &= (\text{Units/ml working Thrombin})(0.2) \\ \text{Excess T units} &= (\text{Units/ml T})(0.3 \text{ Incubation mix}) \end{aligned}$$

0.1 = Volume (in milliliter) of Hirudin used

df = Dilution factor

T units/assay = Thrombin units used in Incubation Mix

Units/ml Working Thrombin = Final thrombin (units/ml) concentration of Thrombin Enzyme solution

0.2 = Volume (in milliliter) of working Thrombin incubated with Hirudin in Incubation Mix

Excess T Units = Thrombin units in Incubation Mix not inhibited by Hirudin

Units/ml T = Measured thrombin concentration of Test mix from

the Thrombin standard curve

0.3 Incubation Mix = Volume (in milliliter) of Test Mix Volume

Units/ml = Thrombin Inhibitor Units per ml of Hirudin

vial H/ml = Hirudin dilution in vials/ml

100 = Percent (%) conversion

Units/vial H = Thrombin Inhibitor Units per vial of Hirudin

Units/ml H = Units/mL Hirudin

UNIT DEFINITION:

One unit will neutralize 1 NIH unit of thrombin at 37°C, based on direct comparison to an NIH thrombin reference standard (Lot J).

FINAL ASSAY CONCENTRATION:

In a 0.30 ml reaction mix, the final concentrations are 33% (v/v) plasma (see thrombin assay), 0.67 unit thrombin,

and 0.33 unit hirudin (components of the enzyme diluent are not included).

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REFERENCE:

Human Blood Coagulation, Homeostasis, and Thrombosis
(1976) pp. 721-722, 2nd ed., R. Biggs, ed., Blackwell
Scientific Publications, Philadelphia, PA

Markwardt, F. (1970) *Methods in Enzymology*, XIX, 924-932

NOTES:

1. The enzymatic assay for thrombin must accompany this assay when it is sent to a customer.
2. Reagent A may be stored in frozen aliquots of 20 ml for up to three years.
3. Reagent C may be stored up to six months at 0 - 5°C.
4. Prepare a minimum of two vials of NHP and combine in a four dram glass vial.
5. Each 10 unit vial contains enough Thrombin for approximately 5 assays. Prepare multiple vials of Thrombin and combine in a four dram glass vial.
6. Concentration of Hirudin should be approximately 10 units/ml. If clotting time of excess thrombin assay is not in the 15 -25 second range, the Hirudin concentration must be adjusted. Dilute Hirudin if the time is greater than 25 seconds. Increase the Hirudin concentration if the time is less than 15 seconds. The Hirudin concentration should be 1/2 of the thrombin concentration in reaction mixture.
7. The most acceptable linear range to measure Hirudin activity is 45 - 55% inhibition of thrombin.
8. This assay is based on the cited reference.
9. 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.