Human Thrombin ELISA Kit

Introduction

Thrombin (activated Factor II [IIa]) is a coagulation protein that has many effects in the coagulation cascade. Thrombin is a serine protease (EC 3.4.21.5) that converts soluble fibrinogen into insoluble strands of fibrin, as well as catalyzing many other coagulation-related reactions (1). Thrombin is in the form of alpha-thrombin that is the immediate end product of prothrombin activation, two further thrombin products can be identified, beta- and gamma-thrombin. These are degraded form that may arise from autodigestion of a thrombin preparation (2, 3).

Principal of the Assay

The Human Thrombin ELISA kit is designed for detection of alpha Thrombin in human cell culture supernatants. This assay employs a quantitative sandwich enzyme immunoassay technique that measures Thrombin in less than 4 hours. A monoclonal antibody specific for Thrombin has been pre-coated onto a microplate. Thrombin in standards and samples is sandwiched by the immobilized antibody and a biotinylated polyclonal antibody specific for Thrombin, which is recognized by a streptavidin-peroxidase conjugate. All unbound material is then washed away and a peroxidase enzyme substrate is added. The color development is stopped and the intensity of the color is measured.

Caution and Warning

- **Prepare all reagents (working diluent buffer, wash buffer, standards, biotinylated-antibody, and SP conjugate) as instructed, prior to running the assay.**
- **Prepare all samples prior to running the assay. The dilution factors for the samples are suggested in this protocol. However, the user should determine the optimal dilution factor.**
- **Spin down the SP conjugate vial and the biotinylated-antibody vial before opening and using contents.**
- This kit is for research use only.
- The kit should not be used beyond the expiration date.
- The Stop Solution is an acid solution

Reagents

- **Thrombin Microplate:** A 96-well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody against alpha Thrombin.
- **Sealing Tapes:** Each kit contains 3 pre-cut, pressure-sensitive sealing tapes that can be cut to fit the format of the individual assay.

- **Thrombin Standard:** Purified human Thrombin in a buffered protein base (40 ng, lyophilized).
- **Biotinylated Thrombin Antibody (80x):** A 80-fold biotinylated polyclonal antibody against Thrombin (100 µl).
- **EIA Diluent Concentrate (10x):** A 10-fold buffered protein base (20 ml).
- **Wash Buffer Concentrate (20x)**: A 20-fold concentrated buffered surfactant (30 ml, 2 bottles).
- **Streptavidin-Peroxidase Conjugate (SP Conjugate):** A 100-fold concentrate (80 µl).
- **Chromogen Substrate**: A ready-to-use stabilized peroxidase chromogen substrate tetramethylbenzidine (8 ml).
- **Stop Solution**: A 0.5 N hydrochloric acid to stop the chromogen substrate reaction (12 ml).

Storage Condition

- Store components of the kit at 2-8^{0}C or -20^{0}C upon arrival up to the expiration date.
- Store SP Conjugate and Biotinylated Antibody at -20^{0}C
- Store Microplate, Diluent Concentrate (10x), Wash Buffer, Stop Solution, and Chromogen Substrate at 2-8^{0}C
- Opened unused microplate wells may be returned to the foil pouch with the desiccant packs. Reseal along zip-seal. May be stored for up to 1 month in a vacuum desiccator.
- Diluent (1x) may be stored for up to 1 month at 2-8^{0}C.
- Store Standard at 2-8^{0}C before reconstituting with Diluent and at -20^{0}C after reconstituting with Diluent.

Other Supplies Required

- Microplate reader capable of measuring absorbance at 450 nm
- Pipettes (1-20 µl, 20-200 µl, 200-1000 µl and multiple channel).
- Deionized or distilled reagent grade water

Sample Collection, Preparation and Storage

- **Cell Culture Supernatants:** Centrifuge cell culture media at 2000 x g for 10 minutes to remove debris. Collect supernatants, dilute if necessary and assay. The samples can be stored at -20^{0}C or below. Avoid repeated freeze-thaw cycles.

Reagent Preparation

- Freshly dilute all reagents and bring all reagents to room temperature before use
- **EIA Diluent Concentrate (10x):** If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved. Dilute the EIA Diluent 1:10 with reagent grade water. Store for up to 1 month at 2-8^{0}C.
- **Standard Curve:** Reconstitute the 40 ng of human Thrombin Standard with 2 ml of EIA Diluent to generate 20 ng/ml of stock solution. Allow the standard to sit for 10 minutes with gentle agitation prior to making dilutions. Prepare duplicate or triplicate standard points by serially diluting the Thrombin standard stock (20 ng/ml) 1:2 with equal volume of EIA Diluent to produce 10, 5, 2.5, 1.25, 0.625 and 0.313 ng/ml. EIA Diluent serves as the zero standard (0 ng/ml). Any remaining solution should be frozen at -20^{0}C.

Standard Point	Dilution	[Alpha Thrombin] (ng/ml)
P1	1 part Standard Stock (20 ng/ml)	20.00
P2	1 part P1 + 1 part EIA Diluent	10.00
P3	1 part P2 + 1 part EIA Diluent	5.000
P4	1 part P3 + 1 part EIA Diluent	2.500
P5	1 part P4 + 1 part EIA Diluent	1.250
P6	1 part P5 + 1 part EIA Diluent	0.625
P7	1 part P6 + 1 part EIA Diluent	0.313
P8	EIA Diluent	0.000

- **Biotinylated Thrombin Antibody (80x):** Spin down the antibody briefly and dilute the desired amount of the antibody 1:80 with EIA Diluent. Any remaining solution should be frozen at -20^{0}C.
- **Wash Buffer Concentrate (20x):** If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved. Dilute the Wash Buffer 1:20 with reagent grade water.
- **SP Conjugate (100x):** Spin down the SP Conjugate briefly and dilute the desired amount of the conjugate 1:100 with EIA Diluent. Any remaining solution should be frozen at -20^{0}C.

Assay Procedure

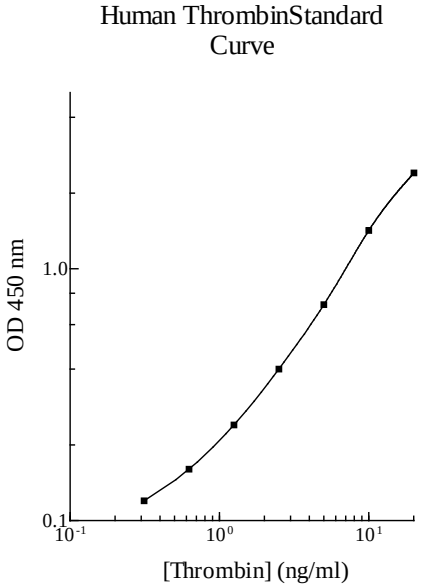
- Prepare all reagents, working standards and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature (20-30^{0}C).
- Remove excess microplate strips from the plate frame and return them immediately to the foil pouch with desiccant inside. Reseal the pouch securely to minimize exposure to water vapor and store in a vacuum desiccator.
- Add 50 µl of Standard or sample per well. Cover wells with a sealing tape and incubate for two hours. Start the timer after the last sample addition.
- Wash five times with 200 µl of Wash Buffer manually. Invert the plate each time and decant the contents; hit it 4-5 times on absorbent paper towel to completely remove the liquid.
  If using a machine wash six times with 300 µl of Wash Buffer and then invert the plate, decant the contents; hit it 4-5 times on absorbent paper towel to completely remove the liquid.
- Add 50 µl of Biotinylated Thrombin Antibody to each well and incubate for one hour.
- Wash the microplate as described above.
- Add 50 µl of Streptavidin-Peroxidase Conjugate per well and incubate for 30 minutes. Turn on the microplate reader and set up the program in advance.
- Wash the microplate as described above.
- Add 50 µl of Chromogen Substrate per well and incubate for approximately 10 minutes or till the optimal blue color density develops. Gently tap the plate to ensure thorough mixing and break the bubbles in the well with pipette tip.
- Add 50 µl of Stop Solution to each well. The color will change from blue to yellow.
- Read the absorbance on a microplate reader at a wavelength of 450 nm **immediately**. If wavelength correction is available, subtract readings at 570 nm from those at 450 nm to correct optical imperfections. Otherwise, read the plate at 450 nm only. Please note that some unstable black particles may be generated at high concentration points after stopping the reaction for about 10 minutes, which will reduce the readings.

Data Analysis

- Calculate the mean value of the duplicate or triplicate readings for each standard and sample. To generate a Standard Curve, plot the graph using the standard concentrations on the x-axis and the corresponding mean 450 nm absorbance on the y-axis. The best-fit line can be determined by regression analysis using log-log or four-parameter logistic curve-fit.
- Determine the unknown sample concentration from the Standard Curve and multiply the value by the dilution factor.

Standard Curve

- The curve is provided for illustration only. A standard curve should be generated each time the assay is performed.



Performance Characteristics

- Intra-assay and inter-assay coefficients of variation were 4.8% and 7.2% respectively.
- The minimum detectable dose of Thrombin is typically ~ 0.3 ng/ml.
- This assay recognizes both natural and recombinant human Thrombin.
- This kit has about 70% cross reactivity to human Prothrombin.

Recovery

Standard Added Value	1 – 10 ng/ml
Recovery %	86-106 %
Average Recovery %	97 %

Cross-Reactivity

Species	% Cross Reactivity
Canine	None
Bovine	None
Monkey	< 20
Mouse	None
Rat	None
Swine	< 3

References

(1) Badimon L *et al.* (1988) *Circulation* 78:1431-1442
(2) Esmon C T *et al.* (1974) *Journal of Biological chemistry* 249: 7798-7807
(3) Hatton M W C *et al.* (1978) *Thrombosis Research* 13: 655-670

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