



# AssayMax Human Thrombin ELISA Kit

Catalog No. ET4010-1

## 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 forms that may arise from autodigestion of a thrombin preparation (2, 3).

## Principal of the Assay

The AssayMax 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 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

- 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 (640 ng, lyophilized).
- **Biotinylated Thrombin Antibody (100x):** A 100-fold biotinylated polyclonal antibody against Thrombin (80  $\mu$ l).
- **EIA Diluent Concentrate (10x):** A 10-fold buffered protein base (30 ml).
- **Wash Buffer Concentrate (20x):** A 20-fold concentrated buffered surfactant (30 ml).
- **Streptavidin-Peroxidase Conjugate (SP Conjugate):** A 100-fold concentrate (90  $\mu$ l).

- **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 kit at 2-8<sup>0</sup>C or -20<sup>0</sup>C upon arrival up to the expiration date.
- Opened EIA Diluent may be stored for up to 1 month at 2-8<sup>0</sup>C. Store reconstituted reagents at -20<sup>0</sup>C or below.
- Opened unused strip wells may return to the foil pouch with the desiccant pack, reseal along zip-seal. May be stored for up to 1 month in a vacuum desiccator.

### 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 and assay. The samples can be stored at -20<sup>0</sup>C or below. Avoid repeated freeze-thaw cycles.

### Reagent Preparation

- Freshly dilute all reagents and bring all reagents to room temperature before use. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.
- **EIA Diluent Concentrate (10x):** Dilute the EIA Diluent 1:10 with reagent grade water. Store for up to 1 month at 2-8<sup>0</sup>C.
- **Standard Curve:** Reconstitute the 640 ng of human Thrombin Standard with 4 ml of EIA Diluent to generate 160 ng/ml of stock solution. Allow the standard to sit for 10 minutes with gentle agitation prior to making dilutions. The stock solution can be further dilute 1:8 with EIA Diluent to produce 20 ng/ml standard solution. Prepare triplicate standard points by serially diluting the Thrombin standard solution 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<sup>0</sup>C.

Standard Point	Dilution	[alpha Thrombin] (ng/ml)
P1	Standard (20 ng/ml)	20.000
P2	1 part P1 + 1 part EIA Diluent	10.000
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 (100x):** Spin down the antibody briefly and dilute the desired amount of the antibody 1:100 with EIA Diluent. Any remaining solution should be frozen at  $-20^{\circ}\text{C}$ .
- **Wash Buffer Concentrate (20x):** 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  $-20^{\circ}\text{C}$ .

## Assay Procedure

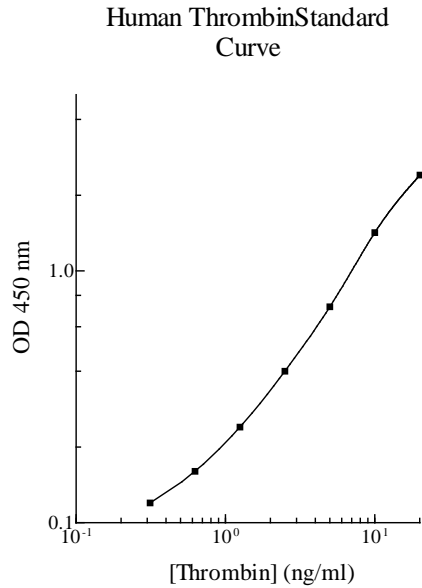
- Prepare all reagents, working standards and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature ( $20\text{-}30^{\circ}\text{C}$ ).
- Remove excess microplate strips from the plate frame and return them immediately to the foil pouch with desiccant inside. Reseal the pouch securely to minimize exposure to water vapor and store in a vacuum desiccator.
- Add  $50\ \mu\text{l}$  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  $200\ \mu\text{l}$  of Wash Buffer. Invert the plate and decant the contents, and hit it 4-5 times on absorbent paper towel to completely remove liquid at each step.
- Add  $50\ \mu\text{l}$  of Biotinylated Thrombin Antibody to each well and incubate for one hour.
- Wash five times with  $200\ \mu\text{l}$  of Wash Buffer as above.
- Add  $50\ \mu\text{l}$  of Streptavidin-Peroxidase Conjugate per well and incubate for 30 minutes. Turn on the microplate reader and set up the program in advance.
- Wash five times with  $200\ \mu\text{l}$  of Wash Buffer as above.
- Add  $50\ \mu\text{l}$  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  $50\ \mu\text{l}$  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. Please note that some unstable black particles may be generated at high optical densities to reduce the readings after stopping the reaction for about 10 minutes.

## Data Analysis

- Calculate the mean value of the 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 5.0% and 7.0% respectively.
- The minimum detectable dose of Thrombin is typically < 300 pg/ml.
- This assay recognizes both natural and recombinant human Thrombin.
- This kit has about 70% cross reactivity to human prothrombin.

## Linearity

	Average Percentage of Expected Value
<b>Sample Dilution</b>	Cell Culture Supernatant
<b>1:10</b>	98%
<b>1:20</b>	105%
<b>1:40</b>	102%

## Recovery

<b>Standard Added Value</b>	0.5 – 10 ng/ml
<b>Recovery %</b>	85-105 %
<b>Average Recovery %</b>	95 %

## Cross-Reactivity

Species	% Cross Reactivity
Beagle	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

Version 3.2

## Related Products

- EP3022-1 AssayMax Human Prothrombin ELISA Kit (Plasma and Cell Culture Supernatants samples)