AssaySense

Human Thrombin Chromogenic Activity Kit

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For any questions regarding troubleshooting or performing the assay, please contact our support team at support@assaypro.com.

Thank you for choosing Assaypro.

Assay Summary

Step 1. Add 10 µl of Standard or Sample per well.
Add 90 µl of Assay Mix per well.

Step 2. Read the absorbance at 405 nm for a zero minute background reading.
Cover and incubate at 37°C.

Step 3. Read the absorbance at 405 nm every 30 minutes for 2 hours.
Cover and incubate at 37°C after each reading.

Symbol Key

Consult instructions for use.

Assay Template

	A	B	C	D	E	F	G	H
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AssaySense Human Thrombin Chromogenic Activity Kit

Catalog No. CT4010

Sample insert for reference use only

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).

Principle of the Assay

The AssaySense Human Thrombin Chromogenic Activity Kit is developed to determine thrombin activity in human **plasma, serum, cell culture supernatant, cell lysate, and tissue samples**. This kit is also validated for use with **canine, bovine, equine, monkey, mouse, rat, swine, and rabbit samples.** The amidolytic activity of thrombin is quantitated using a highly specific thrombin substrate releasing a pNA chromophore. The change in absorbance of the pNA at 405 nm is directly proportional to the thrombin enzymatic activity.

Caution and Warning

- This product is for **Research Use Only** and is not intended for use in diagnostic procedures.
- Prepare all reagents, 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 insert. However, the user should determine the optimal dilution factor.
- The kit should not be used beyond the expiration date.

Reagents

The activity assay kit contains sufficient reagents to perform 96 tests using the microplate method.

- **Microplate:** A 96-well polystyrene microplate (12 strips of 8 wells).
- **Sealing Tapes:** Each kit contains 3 precut, pressure sensitive sealing tapes that can be cut to fit the format of the individual assay.
- **EIA Diluent Concentrate (10x):** A 10-fold concentrated buffered protein base (20 ml).
- **Human Thrombin Standard:** Calibrated against WHO 4th International Standard (0.108 IU, lyophilized).
- **Thrombin Substrate:** Lyophilized, 2 vials.

Storage Condition

- Upon arrival, immediately store components of the kit at recommended temperatures up to the expiration date.
- Store Standard and Thrombin Substrate at -20°C.
- Store Microplate and EIA Diluent Concentrate (10x) at 2-8°C.
- Unused microplate wells may be returned to the pouch and resealed.

Other Supplies Required

- Microplate reader capable of measuring absorbance at 405 nm
- Pipettes (1-20 µl, 20-200 µl, 200-1000 µl, and multiple channel)
- Deionized or distilled reagent grade water
- Incubator (37°C)

Sample Collection, Preparation, and Storage

- **Plasma:** Collect plasma using one-tenth volume of 0.1 M sodium citrate as an anticoagulant. Centrifuge samples at 3000 x *g* for 10 minutes and collect plasma. A 2-fold sample dilution is suggested into EIA Diluent; however, user should determine optimal dilution factor depending on application needs. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles (EDTA or Heparin can also be used as an anticoagulant).
- **Serum:** Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 3000 x *g* for 10 minutes and remove serum. A 20-fold sample dilution is suggested into EIA Diluent; however, user should determine optimal dilution factor depending on application needs. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.
- **Cell Culture Supernatant:** Centrifuge cell culture media at 1500 rpm for 10 minutes at 4°C to remove debris and collect supernatant. If necessary, dilute samples into EIA Diluent; user should determine optimal dilution factor depending on application needs. The undiluted samples can be stored at -80°C. Avoid repeated freeze-thaw cycles.

- **Cell Lysate:** Rinse cell with cold PBS and then scrape the cell into a tube with 5 ml of cold PBS and 0.5 M EDTA. Centrifuge suspension at 1500 rpm for 10 minutes at 4°C and aspirate supernatant. Resuspend pellet in ice-cold Lysis Buffer (PBS, 1% Triton X-100, protease inhibitor cocktail). For every 1 x 10^6 cells, add approximately 100 µl of ice-cold Lysis Buffer. Incubate on ice for 60 minutes. Centrifuge at 13000 rpm for 30 minutes at 4°C and collect supernatant. If necessary, dilute samples into EIA Diluent; user should determine optimal dilution factor depending on application needs. The undiluted samples can be stored at -80°C. Avoid repeated freeze-thaw cycles.
- **Tissue:** Extract tissue samples with 0.1 M phosphate-buffered saline (pH 7.4) containing 1% Triton X-100 and centrifuge at 14000 x *g* for 20 minutes. Collect the supernatant and measure the protein concentration. If necessary, dilute samples into EIA Diluent; user should determine optimal dilution factor depending on application needs. Store remaining extract at -80°C. Avoid repeated freeze-thaw cycles.

Applicable samples may also include biofluids, cell culture, and tissue homogenates. If necessary, user should determine optimal dilution factor depending on application needs.

Refer to Dilution Guidelines for further instruction.

Guidelines for Dilutions of 100-fold or Greater
(*for reference only; please follow the insert for specific dilution suggested)*

100x	**10000x**
A) 4 µl sample : 396 µl buffer (100x) = 100-fold dilution *Assuming the needed volume is less than or equal to 400 µl.*	A) 4 µl sample : 396 µl buffer (100x) B) 4 µl of A : 396 µl buffer (100x) = 10000-fold dilution *Assuming the needed volume is less than or equal to 400 µl.*
1000x	**100000x**
A) 4 µl sample : 396 µl buffer (100x) B) 24 µl of A : 216 µl buffer (10x) = 1000-fold dilution *Assuming the needed volume is less than or equal to 240 µl.*	A) 4 µl sample : 396 µl buffer (100x) B) 4 µl of A : 396 µl buffer (100x) C) 24 µl of B : 216 µl buffer (10x) = 100000-fold dilution *Assuming the needed volume is less than or equal to 240 µl.*

Reagent Preparation

- Freshly dilute all reagents and bring all reagents to room temperature before use.
- **EIA Diluent Concentrate (10x):** Dilute the EIA Diluent Concentrate 10-fold with reagent grade water to produce a 1x solution. When diluting

the concentrate, make sure to rinse the bottle thoroughly to extract any precipitates left in the bottle. Mix the 1x solution gently until the crystals have completely dissolved. Store for up to 30 days at 2-8°C.

- **Human Thrombin Standard:** Reconstitute the Human Thrombin Standard (0.108 IU) with 0.45 ml of EIA Diluent to generate a 0.24 IU/ml standard stock solution. Allow the vial to sit for 10 minutes with gentle agitation prior to making dilutions. Prepare duplicate or triplicate standard points by serially diluting from the standard stock solution (0.24 IU/ml) 2-fold with equal volume of EIA Diluent to produce 0.12, 0.06, 0.03, 0.015, and 0.008 IU/ml solutions. EIA Diluent serves as the zero standard (0 IU/ml). Any remaining stock solution should be stored at -20°C and used within 30 days. Avoid repeated freeze-thaw cycles.

Standard Point	Dilution	[Thrombin] (IU/ml)
P1	1 part Standard (0.24 IU/ml)	0.24
P2	1 part P1 + 1 part EIA Diluent	0.12
P3	1 part P2 + 1 part EIA Diluent	0.06
P4	1 part P3 + 1 part EIA Diluent	0.03
P5	1 part P4 + 1 part EIA Diluent	0.015
P6	1 part P5 + 1 part EIA Diluent	0.008
P7	EIA Diluent	0.0

- **Thrombin Substrate:** Add 1.4 ml of reagent grade water to generate a 1x stock solution. Allow the vial to sit for 10 minutes with gentle agitation prior to use. Any remaining stock solution should be stored at -20°C and used within 30 days. Avoid repeated freeze-thaw cycles.

Assay Procedure

- Prepare all reagents, standard solutions, and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at 37°C in a humid incubator to avoid evaporation.
- Remove excess microplate strips from the plate frame.
- Freshly prepare the desired volume of the Assay Mix by combining the following reagents according to the number of wells in the assay (n) plus one well.

Assay Mix Reagent	n = 1 well
EIA Diluent (1x)	65 µl
Thrombin Substrate	25 µl

- Add 10 µl of Human Thrombin Standard or sample to each well. Gently tap plate to thoroughly coat the wells. Add 90 µl of Assay Mix to each

well. Gently tap plate to ensure thorough mixing. Break any bubbles that may have formed. Read the absorbance at 405 nm for a zero minute background reading. Cover wells with a sealing tape and incubate at 37°C in a humid incubator.

- Read the absorbance at 405 nm every 30 minutes for 2 hours. Cover wells with a sealing tape and incubate at 37°C after each reading.

Data Analysis

- Calculate the mean value of the duplicate or triplicate readings for each standard and sample.
- To generate a standard curve from the optimal reaction time, plot the graph using the standard concentrations on the x-axis and the corresponding mean 405 nm absorbance (OD) or change in absorbance per minute (ΔA/min) on the y-axis after subtracting the background. The best fit line can be determined by regression analysis of the 4-parameter curve.
- Determine the unknown sample concentration from the Standard Curve and multiply the value by the dilution factor.

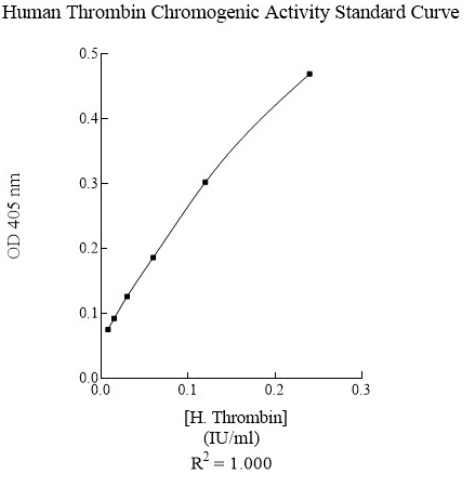
Typical Data

- The typical data is provided for reference only. Individual laboratory means may vary from the values listed. Variations between laboratories may be caused by technique differences.

Standard Point	IU/ml	Average OD
P1	0.24	0.469
P2	0.12	0.302
P3	0.06	0.186
P4	0.03	0.126
P5	0.015	0.092
P6	0.008	0.075
P7	0.0	0.054

Standard Curve

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



Standard Curve at 2 hours

Performance Characteristics

- **Kit standard has been calibrated against WHO International Standard**.
- The minimum detectable dose of human thrombin as calculated by 2SD from the mean of a zero standard was established to be 0.006 IU/ml.

Notes

- The conversion of WHO U and IU is 1 WHO U/ml = 1.2 IU/ml.
- The conversion of AU and IU is 1 AU/ml = 0.15 IU/ml.

Troubleshooting

Issue	Causes	Course of Action
Low Precision	Use of improper components	• Check the expiration date listed before use. • Do not interchange components from different lots.
	Splashing of reagents while loading wells	• Pipette properly in a controlled and careful manner.
	Inconsistent volumes loaded into wells	• Pipette properly in a controlled and careful manner. • Check pipette calibration. • Check pipette for proper performance.

	Insufficient mixing of reagent dilutions	• Thoroughly agitate the lyophilized components after reconstitution. • Thoroughly mix dilutions.
Unexpectedly Low or High Signal Intensity	Microplate was left unattended between steps	• Each step of the procedure should be performed uninterrupted.
	Omission of step	• Consult the provided procedure for complete list of steps.
	Steps performed in incorrect order	• Consult the provided procedure for the correct order.
	Insufficient amount of reagents added to wells	• Check pipette calibration. • Check pipette for proper performance.
	Improper reagent preparation	• Consult reagent preparation section for the correct dilutions of all reagents.
	Insufficient or prolonged incubation periods	• Consult the provided procedure for correct incubation time.
Deficient Standard Curve Fit	Non-optimal sample dilution	• User should determine the optimal dilution factor for samples.
	Contamination of reagents	• A new tip must be used for each addition of different samples or reagents during the assay procedure.
	Contents of wells evaporate	• Verify that the sealing film is firmly in place before placing the assay in the incubator or at room temperature.
	Improper pipetting	• Pipette properly in a controlled and careful manner. • Check pipette calibration. • Check pipette for proper performance.
	Insufficient mixing of reagent dilutions	• Thoroughly agitate the lyophilized components after reconstitution. • Thoroughly mix dilutions.

References

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

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