

Version 3 Last updated 3 December 2021

ab230933 Mouse Thrombin SimpleStep ELISA[®] Kit

For the quantitative measurement of Thrombin in mouse serum, plasma, cell culture supernatants, and tissue extracts.

This product is for research use only and is not intended for diagnostic use.

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1. Overview

Thrombin *in vitro* SimpleStep ELISA® (Enzyme-Linked Immunosorbent Assay) kit is designed for the quantitative measurement of Thrombin protein in mouse serum, plasma, cell culture supernatants, and tissue extracts.

The SimpleStep ELISA® employs an affinity tag labeled capture antibody and a reporter conjugated detector antibody which immunocapture the sample analyte in solution. This entire complex (capture antibody/analyte/detector antibody) is in turn immobilized via immunoaffinity of an anti-tag antibody coating the well. To perform the assay, samples or standards are added to the wells, followed by the antibody mix. After incubation, the wells are washed to remove unbound material. TMB development solution is added and during incubation is catalyzed by HRP, generating blue coloration. This reaction is then stopped by addition of Stop Solution completing any color change from blue to yellow. Signal is generated proportionally to the amount of bound analyte and the intensity is measured at 450 nm. Optionally, instead of the endpoint reading, development of TMB can be recorded kinetically at 600 nm.

Thrombin (activated Factor II [IIa]) is a coagulation protein that has many effects in the coagulation cascade. Thrombin is a serine protease that converts soluble fibrinogen into insoluble strands of fibrin, as well as catalyzing many other coagulation-related reactions. 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. Protocol Summary

Prepare all reagents, samples, and standards as instructed



Add 50 μ L standard or sample to appropriate wells



Add 50 μ L Antibody Cocktail to all wells



Incubate at room temperature for 1 hour



Aspirate and wash each well three times with 350 μ L 1X Wash Buffer
PT



Add 100 μ L TMB Development Solution to each well and incubate
for 10 minutes.



Add 100 μ L Stop Solution and read OD at 450 nm

3. Precautions

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. Storage and Stability

Store kit at +4°C immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components.

5. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. Materials Supplied

| Item | Quantity | Storage Condition |
|---|----------|-------------------|
| Mouse Thrombin Capture Antibody 10X | 600 µL | +4°C |
| Mouse Thrombin Detector Antibody 10X | 600 µL | +4°C |
| Mouse Thrombin Lyophilized Recombinant Protein | 2 Vials | +4°C |
| Antibody Diluent 4BR | 6 mL | +4°C |
| Wash Buffer PT 10X | 20 mL | +4°C |
| Cell Extraction Buffer PTR 5X | 10 mL | +4°C |
| Cell Extraction Enhancer Solution 50X | 1 mL | +4°C |
| TMB Development Solution | 12 mL | +4°C |
| Stop Solution | 12 mL | +4°C |
| Sample Diluent NS | 50 mL | +4°C |
| Anti-tag coated microplate (12 x 8 well strips) | 96 Wells | +4°C |
| Plate Seal | 1 | +4°C |

7. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- Microplate reader capable of measuring absorbance at 450 or 600 nm.
- Method for determining protein concentration (BCA assay recommended).
- Deionized water.
- Multi- and single-channel pipettes.
- Tubes for standard dilution.
- Plate shaker for all incubation steps.
- Optional: Phenylmethylsulfonyl Fluoride (PMSF) (or other protease inhibitors).

8. Technical Hints

- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Complete removal of all solutions and buffers during wash steps is necessary to minimize background.
- As a guide, typical ranges of sample concentration for commonly used sample types are shown below in Sample Preparation (section 11).
- All samples should be mixed thoroughly and gently.
- Avoid multiple freeze/thaw of samples.
- Incubate ELISA plates on a plate shaker during all incubation steps.
- When generating positive control samples, it is advisable to change pipette tips after each step.

- The provided Cell Extraction Enhancer Solution 50X may precipitate when stored at + 4°C. To dissolve, warm briefly at + 37°C and mix gently. The Cell Extraction Enhancer Solution 50X can be stored at room temperature to avoid precipitation.
- **To avoid high background always add samples or standards to the well before the addition of the antibody cocktail.**
- **This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**

9. Reagent Preparation

- Equilibrate all reagents to room temperature (18-25°C) prior to use. The kit contains enough reagents for 96 wells. The sample volumes below are sufficient for 48 wells (6 x 8-well strips); adjust volumes as needed for the number of strips in your experiment.
- Prepare only as much reagent as is needed on the day of the experiment. Capture and Detector Antibodies have only been tested for stability in the provided 10X formulations.

9.1 1X Cell Extraction Buffer PTR (For cell and tissue extracts only):

Prepare 1X Cell Extraction Buffer PTR by diluting Cell Extraction Buffer PTR 5X and 50X Cell Extraction Enhancer Solution to 1X with deionized water. To make 10 mL 1X Cell Extraction Buffer PTR combine 7.8 mL deionized water, 2 mL Cell Extraction Buffer PTR 5X and 200 μ L Cell Extraction Enhancer Solution 50X. Mix thoroughly and gently. If required protease inhibitors can be added.

Alternative – Enhancer may be added to 1X Cell Extraction Buffer PTR after extraction of cells or tissue. Refer to note in the Troubleshooting section.

9.2 1X Wash Buffer PT:

Prepare 1X Wash Buffer PT by diluting Wash Buffer PT 10X with deionized water. To make 50 mL 1X Wash Buffer PT combine 5 mL Wash Buffer PT 10X with 45 mL deionized water. Mix thoroughly and gently.

9.3 Antibody Cocktail:

Prepare Antibody Cocktail by diluting the capture and detector antibodies in Antibody Diluent 4BR. To make 3 mL of the Antibody Cocktail combine 300 μ L 10X Capture Antibody and 300 μ L 10X Detector Antibody with 2.4 mL Antibody Diluent 4BR. Mix thoroughly and gently.

10. Standard Preparation

- Always prepare a fresh set of standards for every use.
- Discard working standard dilutions after use as they do not store well.
- The following section describes the preparation of a standard curve for duplicate measurements (recommended).

10.1 IMPORTANT: If the protein standard vial has a volume identified on the label, reconstitute by adding that volume of dilutant indicated on the label. Alternatively, if the vial has a mass identified, reconstitute by adding 1000 μL of dilutant.

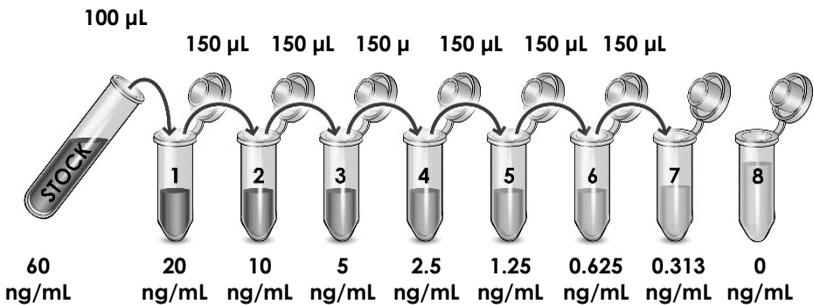
10.2 For **serum, plasmas and cell culture supernatant sample measurements**, reconstitute the Thrombin protein standard in Sample Diluent NS.

10.3 For **tissue extract sample measurements**, reconstitute the Thrombin protein standard in 1X Cell Extraction Buffer PTR. Hold at room temperature for 10 minutes and mix thoroughly and gently. This is the 60 ng/mL **Stock Standard** Solution.

10.4 Label eight tubes, Standards 1– 8.

10.5 Add 200 μL of appropriate diluent (see step 10.1) into tube number 1 and 150 μL of appropriate diluent into numbers 2-8.

10.6 Use the Stock Standard to prepare the following dilution series. Standard #8 contains no protein and is the Blank control:



11. Sample Preparation

| Typical Sample Dynamic Range | |
|------------------------------|------------------------|
| Sample Type | Range |
| Serum | 1:192000 - 1:12000 |
| Plasma - Citrate | 1:16000 - 1:2000 |
| Plasma - EDTA | 1:8000 - 1:500 |
| Plasma - Heparin | 1:8000 - 1:500 |
| 10F RPMI culture media | 12.5% - 50% |
| Mouse liver extract | 62.5 µg/mL - 500 µg/mL |

11.1 Plasma:

Collect plasma using citrate, EDTA or heparin. Centrifuge samples at 2,000 x g for 10 minutes. Dilute samples into Sample Diluent NS and assay. Store un-diluted plasma samples at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

11.2 Serum:

Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 2,000 x g for 10 minutes and collect serum. Dilute samples into Sample Diluent NS and assay. Store un-diluted serum at -20°C or below. Avoid repeated freeze-thaw cycles.

11.3 Cell Culture Supernatants:

Centrifuge cell culture media at 2,000 x g for 10 minutes to remove debris. Collect supernatants and assay. Or dilute samples into Sample Diluent NS and assay. Store un-diluted samples at -20°C or below. Avoid repeated freeze-thaw cycles.

11.4 Preparation of extracts from tissue homogenates:

- 11.4.1 Tissue lysates are typically prepared by homogenization of tissue that is first minced and thoroughly rinsed in PBS to remove blood (dounce homogenizer recommended).
- 11.4.2 Homogenize 100 to 200 mg of wet tissue in 500 µL – 1 mL of chilled 1X Cell Extraction Buffer PTR. For lower amounts of tissue adjust volumes accordingly.
- 11.4.3 Incubate on ice for 20 minutes.

- 11.4.4 Centrifuge at 18,000 x g for 20 minutes at 4°C.
- 11.4.5 Transfer the supernatants into clean tubes and discard the pellets.
- 11.4.6 Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.
- 11.4.7 Dilute samples to desired concentration in 1X Cell Extraction Buffer PTR.

12. Plate Preparation

- The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents.
- Unused plate strips should be immediately returned to the foil pouch containing the desiccant pack, resealed and stored at 4°C.
- For each assay performed, a minimum of two wells must be used as the zero control.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).
- Differences in well absorbance or “edge effects” have not been observed with this assay.

13. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature prior to use.
 - We recommend that you assay all standards, controls and samples in duplicate.
- 13.1** Prepare all reagents, working standards, and samples as directed in the previous sections.
 - 13.2** Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal and return to 4°C storage.
 - 13.3** Add 50 µL of all sample or standard to appropriate wells.
 - 13.4** Add 50 µL of the Antibody Cocktail to each well.
 - 13.5** Seal the plate and incubate for 1 hour at room temperature on a plate shaker set to 400 rpm.
 - 13.6** Wash each well with 3 x 350 µL 1X Wash Buffer PT. Wash by aspirating or decanting from wells then dispensing 350 µL 1X Wash Buffer PT into each well. Wash Buffer PT should remain in wells for at least 10 seconds. Complete removal of liquid at each step is essential for good performance. After the last wash invert the plate and tap gently against clean paper towels to remove excess liquid.
 - 13.7** Add 100 µL of TMB Development Solution to each well and incubate for 10 minutes in the dark on a plate shaker set to 400 rpm.

Given variability in laboratory environmental conditions, optimal incubation time may vary between 5 and 20 minutes.

Note: The addition of Stop Solution will change the color from blue to yellow and enhance the signal intensity about 3X. To avoid signal saturation, proceed to the next step before the high concentration of the standard reaches a blue color of O.D.600 equal to 1.0.
 - 13.8** Add 100 µL of Stop Solution to each well. Shake plate on a plate shaker for 1 minute to mix. Record the OD at 450 nm. This is an endpoint reading.
 - 13.9** Alternative to 13.7 – 13.8: Instead of the endpoint reading at 450 nm, record the development of TMB Substrate kinetically. Immediately after addition of TMB Development Solution begin recording the blue color development with elapsed

time in the microplate reader prepared with the following settings:

| Mode | Kinetic |
|-------------|------------------------|
| Wavelength: | 600 nm |
| Time: | up to 20 min |
| Interval: | 20 sec - 1 min |
| Shaking: | Shake between readings |

Δ Note: that an endpoint reading can also be recorded at the completion of the kinetic read by adding 100 μ L Stop Solution to each well and recording the OD at 450 nm.

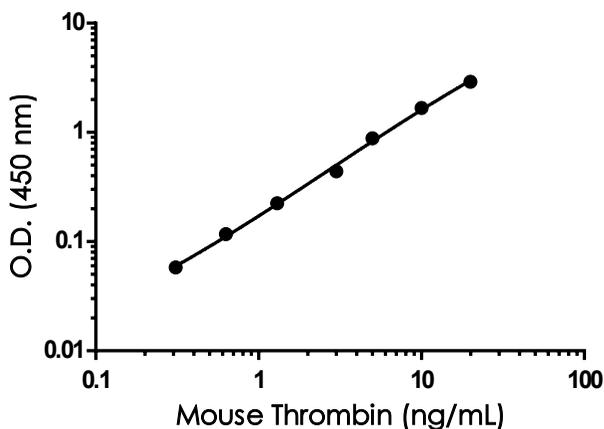
13.10 Analyze the data as described below.

14. Calculations

- 14.1 Calculate the average absorbance value for the blank control (zero) standards. Subtract the average blank control standard absorbance value from all other absorbance values.
- 14.2 **Create a standard curve** by plotting the average blank control subtracted absorbance value for each standard concentration (y-axis) against the target protein concentration (x-axis) of the standard. Use graphing software to draw the best smooth curve through these points to construct the standard curve.
 - Δ **Note:** Most microplate reader software or graphing software will plot these values and fit a curve to the data. A four-parameter curve fit (4PL) is often the best choice; however, other algorithms (e.g. linear, semi-log, log/log, 4-parameter logistic) can also be tested to determine if it provides a better curve fit to the standard values.
- 14.3 Determine the concentration of the target protein in the sample by interpolating the blank control subtracted **absorbance values against the standard curve**. Multiply the resulting value by the appropriate sample dilution factor, if used, to obtain the concentration of target protein in the sample.
- 14.4 Samples generating absorbance values greater than that of the highest standard should be further diluted and reanalyzed. Similarly, samples which measure at an absorbance values less than that of the lowest standard should be retested in a less dilute form.

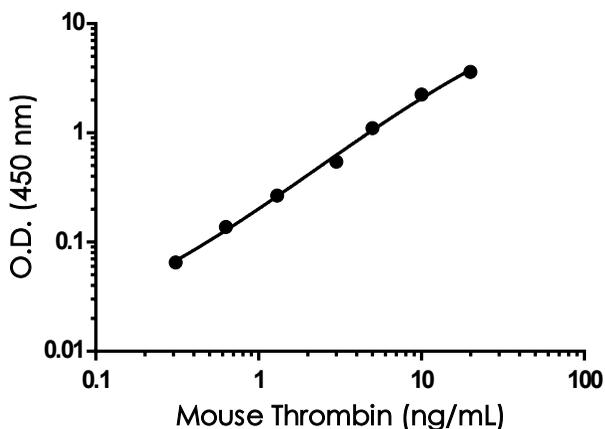
15. Typical Data

Typical standard curve – data provided for demonstration purposes only. A new standard curve must be generated for each assay performed.



| Standard Curve Measurements | | | |
|-----------------------------|------------|-------|----------|
| Concentration (ng/mL) | O.D 450 nm | | Mean O.D |
| | 1 | 2 | |
| 0 | 0.056 | 0.052 | 0.054 |
| 0.31 | 0.114 | 0.110 | 0.112 |
| 0.63 | 0.169 | 0.174 | 0.171 |
| 1.3 | 0.275 | 0.283 | 0.279 |
| 3 | 0.492 | 0.499 | 0.495 |
| 5 | 0.938 | 0.939 | 0.938 |
| 10 | 1.734 | 1.729 | 1.731 |
| 20 | 2.957 | 2.966 | 2.962 |

Figure 1. Example of mouse Thrombin standard curve in Sample Diluent NS. The Thrombin standard curve was prepared as described in Section 10. Raw data values are shown in the table. Background-subtracted data values (mean +/- SD) are graphed.



| Standard Curve Measurements | | | |
|-----------------------------|------------|-------|-------------|
| Concentration (ng/mL) | O.D 450 nm | | Mean O.D |
| | 1 | 2 | |
| 0 | 0.058 | 0.053 | 0.056 |
| 0.31 | 0.122 | 0.120 | 0.121 |
| 0.63 | 0.194 | 0.192 | 0.193 |
| 1.3 | 0.321 | 0.325 | 0.323 |
| 3 | 0.605 | 0.598 | 0.601 |
| 5 | 1.143 | 1.175 | 1.159 |
| 10 | 2.301 | 2.304 | 2.302 |
| 20 | 3.673 | 3.677 | 3.675 |

Figure 2. Example of mouse Thrombin standard curve in 1X Cell Extraction Buffer PTR. The Thrombin standard curve was prepared as described in Section 10. Raw data values are shown in the table. Background-subtracted data values (mean +/- SD) are graphed.

16. Typical Sample Values

SENSITIVITY –

The calculated minimal detectable dose (MDD) is 36 pg/mL for serum, plasma, and cell culture supernatant. The calculated minimal detectable dose (MDD) is 17.5 pg/mL for tissue extracts.

The MDD was determined by calculating the mean of zero standard replicates (n=24) and adding 2 standard deviations then extrapolating the corresponding concentration.

RECOVERY –

Three concentrations of Thrombin mouse recombinant protein were spiked in duplicate to the indicated biological matrix to evaluate signal recovery in the working range of the assay.

| Sample Type | Average % Recovery | Range (%) |
|------------------------------------|--------------------|-----------|
| Serum (1:24000) | 98 | 94-104 |
| Plasma-Citrate (1:4000) | 97 | 91-102 |
| Plasma-EDTA (1:1000) | 108 | 95-117 |
| Plasma-Heparin (1:1000) | 110 | 106-112 |
| 10F RPMI culture media (50%) | 79 | 67-86 |
| Mouse Liver Extract (250 µg/mL) | 99 | 92-103 |

Linearity of Dilution

Linearity of dilution is determined based on interpolated values from the standard curve. Linearity of dilution defines a sample concentration interval in which interpolated target concentrations are directly proportional to sample dilution.

Native Thrombin was measured in the following serum and plasma in 2-fold dilution series. Sample dilutions are made in Sample Diluent NS.

Recombinant Thrombin was spiked into the following 10F RPMI media and diluted in a 2-fold dilution series in Sample Diluent NS.

Native Thrombin was measured in mouse liver extract in a 2-fold dilution series. Sample dilutions are made in 1X Cell Extraction Buffer PTR.

| Dilution Factor | Interpolated value | 1:12000 Mouse Serum | 1:2000 Mouse Plasma (Citrate) | 1:500 Mouse Plasma (EDTA) | 1:500 Mouse Plasma (Heparin) | 50% 10F RPMI (Spiked) | Mouse Liver Extract (500 µg/mL) |
|-----------------|-------------------------|---------------------|-------------------------------|---------------------------|------------------------------|-----------------------|---------------------------------|
| Undiluted | ng/mL | 3.33 | 1.94 | 11.83 | 10.05 | 9.08 | 2.60 |
| | % Expected value | 100 | 100 | 109 | 100 | 100 | 100 |
| 2 | ng/mL | 1.62 | 0.89 | 5.51 | 4.71 | 4.74 | 1.17 |
| | % Expected value | 97 | 93 | 101 | 94 | 104 | 90 |
| 4 | ng/mL | 0.76 | 0.41 | 2.53 | 2.19 | 2.29 | 0.53 |
| | % Expected value | 91 | 83 | 93 | 87 | 101 | 81 |
| 8 | ng/mL | 0.34 | 0.26 | 1.30 | 1.01 | 1.26 | 0.26 |
| | % Expected value | 81 | 96 | 96 | 80 | 111 | 81 |
| 16 | ng/mL | 0.20 | NL | 0.66 | 0.53 | NL | NL |
| | % Expected value | 94 | NL | 98 | 84 | NL | NL |

NL – Non-Linear

PRECISION –

Mean coefficient of variations of interpolated values of Thrombin from one concentration of mouse serum within the working range of the assay.

| | Intra- Assay | Inter- Assay |
|-------|-----------------|-----------------|
| n = | 8 | 3 |
| CV(%) | 3.2 | 6.3 |

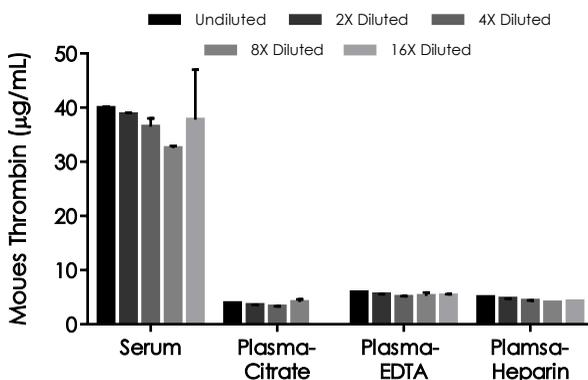


Figure 3. Interpolated concentrations of native Thrombin in mouse serum and plasma. The concentrations of Thrombin were measured in duplicate, interpolated from the Thrombin standard curves and corrected for sample dilution. Undiluted samples are as follows: serum 1:12000, plasma (citrate) 1:2000, plasma (EDTA) 1:500, and plasma (heparin) 1:500. The interpolated dilution factor corrected values are plotted (mean +/- SD, n=2). The mean Thrombin concentration was determined to be 37.1 µg/mL in serum, 3.7 µg/mL in plasma (citrate), 5.4 µg/mL in plasma (EDTA), and 4.48 µg/mL in plasma (heparin).

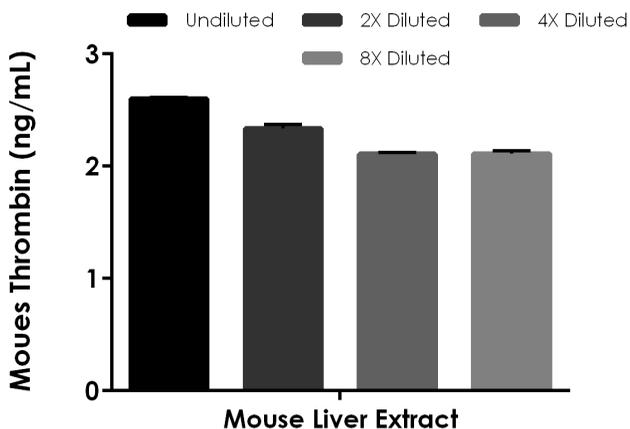


Figure 4. Interpolated concentrations of native Thrombin in mouse liver extract based on a 500 µg/mL extract load. The concentrations of Thrombin were measured in duplicate and interpolated from the Thrombin standard curve and corrected for sample dilution. The interpolated dilution factor corrected values are plotted (mean +/- SD, n=2). The mean Thrombin concentration was determined to be 2.29 ng/mL in mouse liver extract.

17. Assay Specificity

This kit recognizes both native and recombinant mouse Thrombin protein in serum, plasma, and cell culture supernatant, cell and tissue extract samples only.

CROSS REACTIVITY

Recombinant rat Thrombin was prepared at 350 ng/mL along with serum and plasma biologicals in the linear range and assayed for cross reactivity. Cross-reactivity with rat Thrombin was observed.

INTERFERENCE

Recombinant Antithrombin III and was prepared at 20 ng/mL and diluted 2-fold and tested for interference. No interference was observed.

18. Species Reactivity

This kit recognizes mouse Thrombin protein.

Other species reactivity was determined by measuring 1:8000 rat or human serum samples, interpolating the protein concentrations from the mouse standard curve, and expressing the interpolated concentrations as a percentage of the protein concentration in mouse serum assayed at the same dilution.

Rat serum was determined to be 173% cross reactive with mouse serum.

Reactivity < 3% was determined for the following species:

- Human

Please contact our Technical Support team for more information.

19. Troubleshooting

| Problem | Reason | Solution |
|---|--|--|
| Difficulty pipetting lysate; viscous lysate. | Genomic DNA solubilized | Prepare 1X Cell Extraction Buffer PTR (without enhancer). Add enhancer to lysate after extraction. |
| Poor standard curve | Inaccurate Pipetting | Check pipettes |
| | Improper standard dilution | Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing |
| Low Signal | Incubation times too brief | Ensure sufficient incubation times; increase to 2 or 3 hour standard/sample incubation |
| | Inadequate reagent volumes or improper dilution | Check pipettes and ensure correct preparation |
| | Incubation times with TMB too brief | Ensure sufficient incubation time until blue color develops prior addition of Stop solution |
| Large CV | Plate is insufficiently washed | Review manual for proper wash technique. If using a plate washer, check all ports for obstructions. |
| | Contaminated wash buffer | Prepare fresh wash buffer |
| Low sensitivity | Improper storage of the ELISA kit | Store your reconstituted standards at -80°C, all other assay components 4°C. Keep TMB substrate solution protected from light. |
| Precipitate in Diluent | Precipitation and/or coagulation of components within the Diluent. | Precipitate can be removed by gently warming the Diluent to 37°C. |

Technical Support

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