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# Thrombin Generation Assay

## Kit Insert



[www.Haemoscan.com](http://www.Haemoscan.com)

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## Summary

Thrombin is a key enzyme of the coagulation cascade. Its measurement gives direct information about the thrombogenicity of a biomaterial (i.e., its ability to form blood clots). In normal plasma, thrombin is captured into the fibrin meshwork and is rapidly inactivated by antithrombin III or other antiproteases. The short half-life of thrombin hampers its accurate enzymatic determination. Therefore, HaemoScan has designed the Thrombin Generation Assay [1], which is based on a special plasma product that enables the determination of thrombin activity in an incubation medium after this has been exposed to a biomaterial. This method is suited to evaluate the haemocompatibility of biomaterials and medical devices according to the international standard ISO 10993-4:2002.

[1] W. van Oeveren, J. Haan, P. Lagerman, and P. Schoen, "Comparison of coagulation activity tests in vitro for selected biomaterials.," *Artificial organs*, vol. 26, no. 6, pp. 506–11, Jun. 2002.

## Introduction

Interactions between blood components and biomaterials may activate the clotting cascade. Activated factor XII, the Hageman factor (FXIIa) initiates the intrinsic pathway. Depending on the surface properties of the biomaterial the prekallikrein and high-molecular weight kininogen system accelerate FXII activation. Downstream, this results in activation of Factor XI (FXIa), Factor IX (FIXa) and Factor X (FXa). Finally, FXa activates prothrombin to thrombin, which converts fibrinogen into fibrin to form a fibrin meshwork.

Thrombin will also activate platelets, Factor VIII (FVIIIa) and Factor V (FVa). Activated platelets, which expose negatively charged phospholipid, further enhance coagulation by supporting the formation of FIXa-FVIIIa and Fxa-FVa complexes that dramatically increase the rate of thrombin generation. After initial fibrin clot formation, ongoing thrombin generation is required to sustain and stabilize the clot.

## Principle of the Test

The TGA gives an indication of the thrombogenicity of a biomaterial. The amount and speed of thrombin formation, when the biomaterial is incubated in modified plasma, is measured and compared to that of reference materials. Highly reactive and less-reactive reference materials are included in the kit, one type of metal and two types of polymers. It is

recommended to include at least two reference materials in each analysis. Reference materials can be selected to compare with the activities of the test samples.

Materials are first incubated in plasma. After the TGA reagent has been added, the generation of thrombin will begin. At different time points the thrombin generation is measured. The concentration of thrombin is determined by enzymatic reaction with a thrombin-specific chromogenic substrate, which yields a yellow colored product proportional to the amount of thrombin.

The thrombin concentrations of the samples can be determined from a calibration curve. A thrombin generation curve for each material is constructed by plotting the thrombin concentrations versus the time points on which the samples were taken. This curve is used to determine the speed of thrombin generation, which is expressed per  $\text{cm}^2$  sample. The results of the tested materials in relation to the reference materials may be used to evaluate the thrombogenicity. It must be noted that pass/fail criteria cannot be applied yet since criteria for this evaluation method are not available.

The kit is designed to determine thrombin-generating activity of small samples of biomaterial. Larger samples can also be used as long as the ratio between TGA plasma and material size is respected.

## **Precautions**

- The kit is intended for research use only.
- The kit should not be used beyond its expiration date.
- Do not combine reagents from TGA Kits with different lot numbers.
- The plasma is of human origin and has been tested and confirmed negative for HbsAg, HCV, HIV I/II, HTLV I/II and Treponema. However, the plasma should always be treated as a potential biohazard during use and for disposal.
- Chemicals and reagents have to be treated as hazardous waste according to biohazard safety guidelines or regulations. For information on hazardous substances included in the kit please refer to the Material Safety Data Sheets, which are available upon request.
- Wear disposable (latex) gloves when handling specimens and reagents.
- Never pipette by mouth and avoid contact of skin and mucous membranes with reagents containing sodium azide, the Substrate and Stop Solution. Wash thoroughly with large amount of water if

contact occurs.

- Use disposable pipette tips throughout the procedure to avoid contamination of reagents.

## Contents of the Kit

• Thrombin, 7.5 Units	75 $\mu$ L	2 vials
• Buffer A, Phosphate buffer with BSA, 0.01% sodium azide	11 mL	1 bottle
• TGA Plasma, modified human plasma	750 $\mu$ L	5 vials
• Saline, NaCl solution, 0.01% sodium azide	5 mL	1 bottle
• TGA Reagent A, contains 0.01% sodium azide	5 mL	2 bottles
• TGA Reagent B, contains 0.01% sodium azide	600 $\mu$ L	2 vials
• Buffer B, Tris buffer, 0.01% sodium azide	30 mL	2 bottles
• Substrate, Thrombin-specific chromogenic substrate	0.6 mL	2 vials
• Stop Solution, Acetic acid (20%)	6 mL	1 bottle
• Reference 1, Low-density polyethylene (LDPE)	0.6 cm <sup>2</sup>	5 pcs
• Reference 2, Polydimethylsiloxane (PDMS)	0.7 cm <sup>2</sup>	5 pcs
• Reference 3, Medical steel (MS)	0.6 cm <sup>2</sup>	5 pcs

## Additional Materials and Equipment

The following materials and equipment are required but are not provided with the kit:

- (Calibrated) adjustable pipettes with disposable tips.
- Water bath at 37 °C.
- Incubator at 37 °C.
- Timer.
- Crushed ice.
- Plate reader capable of measuring at 405 and 540 nm wavelengths.
- Tweezers.
- Mixer.
- Micro-centrifuge vials (1.5 mL), test tubes (10 mL)

- Optional, for cleaning of test samples:
  - RBS (chlorinated trisodium phosphate, sodium metasilicate) or a comparable detergent.
  - Sonicator.
  - Ultra pure water.

## Test Procedure

### Preparation of Materials for Testing

The TGA test can be used for coated or uncoated biomaterials. It is recommended to test clean samples. The following procedure is recommended to clean biomaterials:

1. Sonicate the biomaterial for 15 min in 2% RBS and wash three times with ultra pure water.
2. Sonicate the biomaterial for 5 min in 70% ethanol and wash three times with ultra pure water.
3. Dry the material in the air.

### **NOTES:**

- Cleaned materials should always be handled with tweezers.
- This cleaning procedure is a recommendation only; each user should determine an own optimal procedure.
- The reference materials provided in the kit are clean and ready for use.

### Reagent Preparation

Select the required reagents and number of multi-well strips. The volumes given in the procedure are based on biomaterial pieces of 0.5 x 0.5 cm (surface area approximately 0.6 cm<sup>2</sup>).

Keep Thrombin and TGA reagent B on ice until use. Allow all other reagents to reach room temperature prior to use.

After first use, Buffer A, Buffer B, Saline and Stop Solution can be stored at 2-8 °C.

Prepare reagents as follows:

- **TGA Plasma:** Dilute TGA plasma by adding 750 µL Saline per vial of TGA plasma, while it is thawing. Mix gently; do not shake. After reconstitution the TGA plasma must be stored on ice and used within 0,5 h. Each vial is sufficient for 4 biomaterial samples.
- **Substrate solution:** Add 2.4 mL of Buffer B to each vial of

substrate. Prepare freshly prior to use. The volume per vial is sufficient for 48 wells of a 96-well microtitre plate.

- **Thrombin calibrators:** Dilute 20  $\mu\text{L}$  of Thrombin with 1980  $\mu\text{L}$  Buffer A in a separate vial. Use the latter dilution to prepare Thrombin calibrators according to Table 1. First pipette Buffer A into the vials and then the volume of diluted Thrombin. Use Buffer A as a blank (BLK). Prepare calibrators freshly each day and store on ice till further use.

Table 1. Preparation of Thrombin Calibrators.

	Thrombin Concentration [mU/mL]	Buffer A [ $\mu\text{L}$ ]	Diluted Thrombin [ $\mu\text{L}$ ]
BLK	0	1000	0
CAL1	25	975	25
CAL2	50	950	50
CAL3	100	900	100
CAL4	150	850	150
CAL5	200	800	200
CAL6	300	700	300
CAL7	400	600	400

## Assay Procedure

### **NOTES IN ADVANCE:**

- Never use pipettes or vials of glass since glass is a material that may substantially activate the clotting system.
- Select 2 or 3 types of reference materials to which the activities of the test materials can be compared. As a guideline Reference 1 (LDPE) should be considered as minimally reactive, Reference 2 (PDMS) as intermediary reactive and Reference 3 (MS) as highly reactive.
- The reference materials provided in the kit have been cleaned by the protocol described above and are ready for use.
- The volumes given in this procedure are based on pieces of material of 0.5 x 0.5 cm (surface area approximately 0.6  $\text{cm}^2$ ). Test materials should be prepared in pieces of approximately the same size. A correction for surface area is made in the final calculations.
- It is important to take samples at the exact time points as described below. Since samples have to be taken at timed intervals it is recommended that materials and controls be incubated as described below in sets of no more than two with a 30-sec interval.

1. Place the selected reference and (cleaned) test materials in micro-centrifuge vials using tweezers. Fix the materials vertically between the walls of the vials to prevent floating. Use vials without material as a negative control (CTRL).
2. Add 350  $\mu\text{L}$  (diluted) TGA plasma to each vial with specimen (test material, reference material or negative control). Be sure that the biomaterial is completely immersed in the plasma.
3. Incubate the vial(s) for 15 min in a water bath at 37  $^{\circ}\text{C}$ .
4. Directly after starting the TGA plasma incubation, also incubate a vial containing 450  $\mu\text{L}$  of TGA Reagent A + 50  $\mu\text{L}$  of TGA Reagent B.
5. During the 15 min incubation of the TGA plasma and the Reagent A + Reagent B mixture, prepare for each specimen (test material, reference material or negative control) 4 labeled micro-centrifuge vials (SPL1-4), each containing 490  $\mu\text{L}$  Buffer B and place the vials on ice.
6. Add, at  $t= 15$  min, 150  $\mu\text{L}$  of the mixture of TGA Reagent A and B to the vial with plasma and mix gently.
7. At  $t= 16$  min, collect a sample of 10  $\mu\text{L}$  out of the reaction mixture into the vial labeled SPL1, which already contains 490  $\mu\text{L}$  Buffer B, vortex and store on ice ( $t= 1$  min). Mix the vials with reaction medium just before removing the 10 $\mu\text{L}$  samples and immediately place the vials back into the water bath after sampling.
8. Repeat this sampling procedure at  $t= 17$  min,  $t= 18$  min and  $t= 19$  min to collect SPL2, SPL3 and SPL4, respectively.
9. Keep all diluted samples on ice until all test materials, reference materials and controls have been processed. Samples can be stored on ice for up to 2 h. For each additional material or control, start again at step no 2.
10. Remove all calibrators and diluted samples collected in Buffer B from the ice, vortex and place at room temperature.
11. Transfer 150  $\mu\text{L}$  of each calibrator and diluted sample to the 96-well plate. It is recommended to pipette each sample in duplicate. Table 2 illustrates a template that might be used.
12. Incubate the plate for 2 min in an incubator at 37  $^{\circ}\text{C}$ .
13. Add 50  $\mu\text{L}$  of diluted Substrate solution to each well (use of a repetition pipette is highly recommended; pipette in the same order as initially used to fill the plate).
14. Cover the plate with a plate cover or foil and incubate for 20 min at 37  $^{\circ}\text{C}$ .
15. Following the incubation, add 50  $\mu\text{L}$  of Stop Solution to each well. Stop Solution should be added to the wells in the same sequence as the Substrate was added (use of a repetition pipette is highly recommended).

16. Read the optical density at 405 nm (OD405) and 540 nm (OD540). The reading has to be performed within 30 min after the Stop Solution was added.
17. Calculate the thrombin concentration of all diluted samples and calculate the velocity of thrombin generation for each test material, reference material and negative control (see Calculations).

Table 2. Suggested 96-well template for the TGA assay.

	1	2	3	4	5	6	7	8	9	10	11	12
<b>A</b>	BLK	BLK	CTRL SPL1	CTRL SPL1	REF2 SPL1	REF2 SPL1	TST2 SPL1	TST2 SPL1	TST4 SPL1	TST4 SPL1	TST6 SPL1	TST6 SPL1
<b>B</b>	CAL 1	CAL 1	CTRL SPL2	CTRL SPL2	REF2 SPL2	REF2 SPL2	TST2 SPL2	TST2 SPL2	TST4 SPL2	TST4 SPL2	TST6 SPL2	TST6 SPL2
<b>C</b>	CAL 2	CAL 2	CTRL SPL3	CTRL SPL3	REF2 SPL3	REF2 SPL3	TST2 SPL3	TST2 SPL3	TST4 SPL3	TST4 SPL3	TST6 SPL3	TST6 SPL3
<b>D</b>	CAL 3	CAL 3	CTRL SPL4	CTRL SPL4	REF2 SPL4	REF2 SPL4	TST2 SPL4	TST2 SPL4	TST4 SPL4	TST4 SPL4	TST6 SPL4	TST6 SPL4
<b>E</b>	CAL 4	CAL 4	REF1 SPL1	REF1 SPL1	TST1 SPL1	TST1 SPL1	TST3 SPL1	TST3 SPL1	TST5 SPL1	TST5 SPL1	TST7 SPL1	TST7 SPL1
<b>F</b>	CAL 5	CAL 5	REF1 SPL2	REF1 SPL2	TST1 SPL2	TST1 SPL2	TST3 SPL2	TST3 SPL2	TST5 SPL2	TST5 SPL2	TST7 SPL2	TST7 SPL2
<b>G</b>	CAL 6	CAL 6	REF1 SPL3	REF1 SPL3	TST1 SPL3	TST1 SPL3	TST3 SPL3	TST3 SPL3	TST5 SPL3	TST5 SPL3	TST7 SPL3	TST7 SPL3
<b>H</b>	CAL 7	CAL 7	REF1 SPL4	REF1 SPL4	TST1 SPL4	TST1 SPL4	TST3 SPL4	TST3 SPL4	TST5 SPL4	TST5 SPL4	TST7 SPL4	TST7 SPL4

### Calculations\*

1. Plot OD405 (corrected for OD540) against the thrombin concentrations of the calibrators. The calibration curve must be a straight line ( $OD405 = A \times CAL + B$ ).
2. Interpolate the thrombin concentration of all samples for all sampling time points.
3. Construct for all test and reference materials and all controls a thrombin generation curve, in which the concentration of thrombin (mU/mL) is plotted against the incubation time.
4. Correct, for all test and reference materials, the thrombin concentration at each time point for the corresponding thrombin concentration of the negative control(s).
5. Determine for the test and reference materials and the negative control the highest velocity of thrombin generation ( $\Delta mU/mL/min$ ) in between 2 measured time points (i.e., at the point of the steepest slope on the curve) and correct this value for the dilution factor (50x).



6. Calculate the thrombin generation of the biomaterials and reference materials in  $\Delta\text{mU/mL/min/cm}^2$ . Reference 1 has a surface area of  $0.6\text{ cm}^2$ , Reference 2 of  $0.7\text{ cm}^2$  and Reference 3 of  $0.6\text{ cm}^2$ .

**\*Upon request an electronic file can be provided to perform the calculations.**

## Assay Criteria

- The correlation coefficient of the calibration curve should be  $\geq 0.98$ .
- The parameters A and B of the calibration curve must be in between the values as given in the 'Certificate of Analysis' (enclosed with the kit).
- The result of the negative control should be  $\leq 2000\text{ }\Delta\text{mU/mL/min}$ .
- The results of the reference materials should be in the ranges as provided in the 'Certificate of Analysis'.
- Repeat the TGA procedure if the results do not meet the assay criteria.

## Characteristics

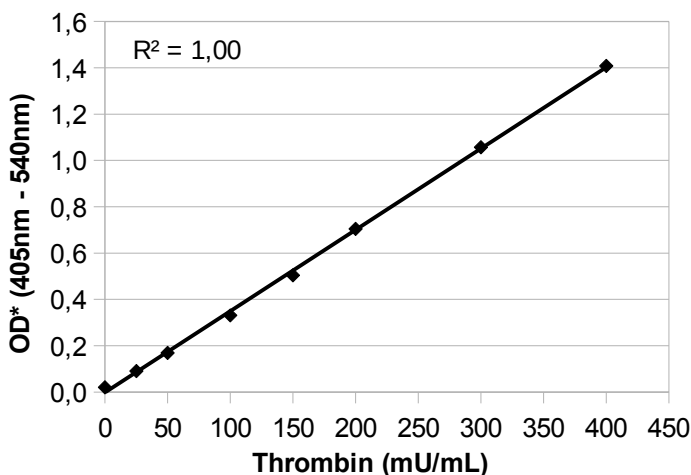


Figure 1. Example of a thrombin calibration curve. This curve is an example only and should not be used for calculations.

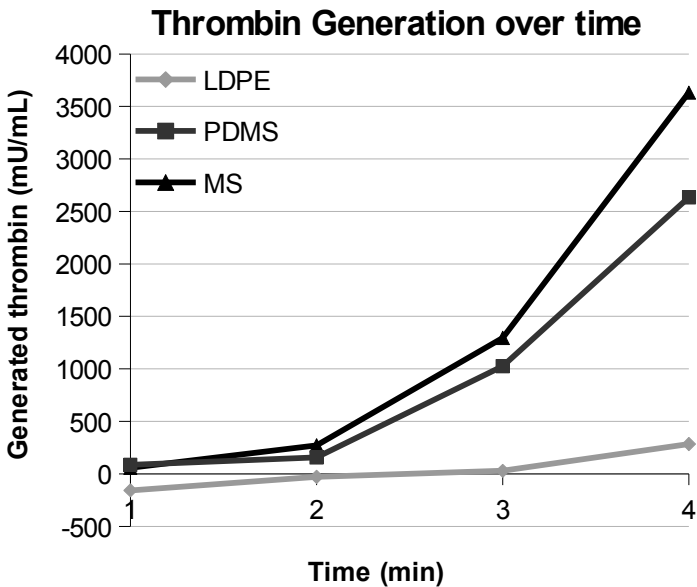


Figure 2. Typical thrombin generation curves for low-density polyethylene (LDPE), polydimethylsiloxane (PDMS) and medical steel (MS).

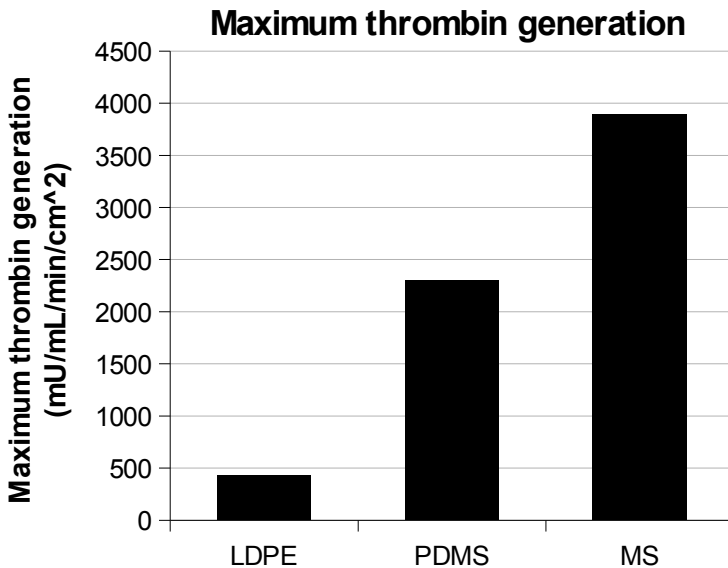


Figure 3. Typical maximum thrombin generation patterns for Low-density polyethylene (LDPE), polydimethylsiloxane (PDMS) and medical steel (MS).