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Thrombin Generation Assay

Kit Manual

www.HaemoScan.com

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Summary

Thrombin is a key enzyme of the coagulation cascade. Its measurement provides direct information on the thrombogenicity of a biomaterial, i.e. its propensity to induce blood clot formation. In normal plasma, thrombin is bound to the fibrin network and is rapidly inactivated by antithrombin and other protease inhibitors. This short half-life hampers accurate enzymatic determination of thrombin. HaemoScan has therefore developed the Thrombin Generation Assay (TGA) [1], which is based on a special plasma product that allows thrombin activity to be determined in an incubation medium after exposure to a biomaterial. The method is suitable for evaluating the haemocompatibility of biomaterials and medical devices in accordance with ISO 10993-4.

[1] van Oeveren W, Haan J, Lagerman P, Schoen P. Comparison of coagulation activity tests in vitro for selected biomaterials. *Artif Organs*. 2002;26(6):506–511.

Introduction

Interactions between blood components and biomaterials may activate the coagulation cascade. Activated factor XII (FXIIa; Hageman factor) initiates the intrinsic (contact activation) pathway. Depending on the surface properties of the biomaterial, prekallikrein and high-molecular-weight kininogen accelerate FXII activation. Downstream, this results in the activation of factor XI (FXIa), factor IX (FIXa) and factor X (FXa). Finally, FXa converts prothrombin into thrombin, which converts fibrinogen into fibrin to form a fibrin network.
Thrombin also activates platelets, factor V (FVa) and factor VIII (FVIIIa). Activated platelets expose negatively charged phospholipids, which further enhance coagulation by supporting the formation of the FIXa–FVIIIa (tenase) and FXa–FVa (prothrombinase) complexes; these complexes dramatically increase the rate of thrombin generation. After initial fibrin clot formation, ongoing thrombin generation is required to sustain and stabilise the clot.

Principle of the Test

The TGA gives an indication of the thrombogenicity of a biomaterial. The amount and rate of thrombin formation when the biomaterial is incubated in modified plasma are measured and compared with those of reference materials. Reference materials with high and low reactivity are included in the kit: one metal and two polymers. It is recommended to include at

least two reference materials in each analysis; these can be selected to suit the test samples.

Materials are first incubated in plasma. After addition of the TGA reagent, thrombin generation begins, and it is measured at several time points. The thrombin concentration is determined by an enzymatic reaction with a thrombin-specific chromogenic substrate, which yields a yellow product in proportion to the amount of thrombin.
The thrombin concentrations of the samples are determined from a calibration curve. A thrombin generation curve is constructed for each material by plotting the thrombin concentrations against the sampling times. This curve is used to determine the rate of thrombin generation, which is expressed per cm^2 of sample surface. The results of the test materials relative to those of the reference materials may be used to evaluate thrombogenicity. Note that pass/fail criteria cannot yet be applied, as no criteria have been established for this evaluation method.

The kit is designed to determine the thrombin-generating activity of small biomaterial samples. Larger samples can also be used, provided that the ratio between the volume of TGA plasma and the size of the material is maintained.

Precautions

- The kit is intended for research use only. It is not intended for diagnostic or therapeutic procedures.
- Store the kit at −20 °C.
- Do not use the kit beyond its expiry date.
- Do not combine reagents from TGA kits with different lot numbers.
- The TGA plasma and the Thrombin are of human origin. The donor blood was tested and found negative for anti-HIV-1/2, HBsAg, anti-HCV, syphilis, HIV-1 RNA, HBV DNA and HCV DNA. As no test method can completely exclude the presence of infectious agents, handle these components as potentially infectious material (biosafety level 2). Some components contain protein of bovine origin.
- Dispose of the kit components and contaminated materials as biological (potentially infectious) waste in accordance with local and national regulations. For further information, please refer to the Safety Data Sheet (SDS), available at www.haemoscan.com.
- Wear disposable nitrile gloves, a laboratory coat and safety glasses.
- Avoid contact of reagents with skin and mucous membranes. In case of contact, rinse thoroughly with plenty of water.

Contents of the Kit

• Thrombin (human), 7.5 U	75 µL	2 vials
• Buffer A, phosphate buffer with BSA	11 mL	1 bottle
• TGA plasma, modified human plasma (freeze-dried)	37.5 mg	5 vials
• Saline, NaCl solution	5 mL	1 bottle
• TGA reagent A	5 mL	2 bottles
• TGA reagent B	600 µL	2 vials
• Buffer B, Tris buffer	30 mL	2 bottles
• Substrate, thrombin-specific chromogenic substrate	0.6 mL	2 vials
• Stop solution (acidic aqueous solution)	6 mL	1 bottle
• Reference 1, low-density polyethylene (LDPE)	0.6 cm^2	5 pieces
• Reference 2, polydimethyl-siloxane (PDMS)	0.7 cm^2	5 pieces
• Reference 3, medical-grade steel (MS)	0.6 cm^2	5 pieces

Additional Materials and Equipment

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

- Calibrated adjustable pipettes with disposable tips and a repeater pipette
- Water bath at 37 °C
- Incubator at 37 °C
- Timer
- Crushed ice
- Microplate reader capable of measuring at 405 and 540 nm
- 96-well flat-bottom microplate and plate cover or sealing film
- Tweezers
- Vortex mixer
- Microcentrifuge tubes (1.5 mL) and test tubes (10 mL)

- Optional, for cleaning of test samples:
  - RBS 35 (Carl Roth) or a comparable laboratory detergent
  - Sonicator
  - Ultrapure water and 70% ethanol

Test Procedure

Preparation of Materials for Testing

The TGA can be used for coated or uncoated biomaterials. It is recommended that clean samples are tested. The following procedure is recommended for cleaning biomaterials:

1. Sonicate the biomaterial for 15 minutes in 2% RBS and wash three times with ultrapure water.
2. Sonicate the biomaterial for 5 minutes in 70% ethanol and wash three times with ultrapure water.
3. Allow the material to air-dry.

Notes:

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

Reagent Preparation

Select the required reagents and number of microplate strips. The volumes given in the procedure are based on biomaterial pieces of 0.5 × 0.5 cm (surface area approximately 0.6 cm^2).

Keep the Thrombin, TGA reagent B and Saline on ice until use. Allow all other reagents to reach room temperature before use.

After first use, Buffer A, Buffer B, Saline and Stop solution can be stored at 2–8 °C. Re-use of TGA reagent A, TGA reagent B and Substrate is not recommended.

Prepare reagents as follows:

- **TGA plasma:** Reconstitute one vial of freeze-dried plasma with 750 µL of cold ultrapure water. Leave on ice for 10 minutes and mix by end-over-end inversion. Then dilute by adding 750 µL of cold Saline. After reconstitution and dilution, keep the TGA plasma on ice and use it within 30 minutes. Each vial is sufficient for 4 biomaterial samples.
- **Substrate solution:** Add 2.4 mL of Buffer B to each vial of Substrate. Prepare freshly before use. Each vial is sufficient for 48 wells of a 96-well microplate.

- **Thrombin calibrators:** Dilute 20 µL of Thrombin with 1980 µL of Buffer A in a separate tube (1000 mU/mL). Use this dilution to prepare the thrombin calibrators according to Table 1: first pipette the Buffer A into the tubes, then add the diluted Thrombin. Use Buffer A as the blank (BLK). Prepare the calibrators freshly each day and keep them on ice until use.

Table 1. Preparation of thrombin calibrators.

	Thrombin (mU/mL)	Buffer A (µL)	Diluted Thrombin (µL)
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 glass pipettes or glass tubes, since glass may substantially activate the coagulation system.
- Select 2 or 3 reference materials with which the activities of the test materials can be compared.
- The reference materials provided in the kit have been cleaned according to the protocol described above and are ready for use.
- The volumes given in this procedure are based on pieces of material of 0.5 × 0.5 cm (surface area approximately 0.6 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 exactly the time points described below. Because samples must be taken at timed intervals, it is recommended to incubate materials and controls in sets of no more than two, started 30 seconds apart.

1. Using tweezers, place the selected reference and (cleaned) test materials in microcentrifuge tubes. Wedge the materials vertically between the walls of the tubes to prevent them from floating. Use tubes without material as the negative control (CTRL).
2. Add 350 µL of (diluted) TGA plasma to each tube (test material, reference material or negative control). Ensure that the biomaterial is completely immersed in the plasma.
3. Incubate the tube(s) for 15 minutes in a water bath at 37 °C.
4. Immediately after starting the incubation of the TGA plasma, also incubate a tube containing 450 µL of TGA reagent A and 50 µL of TGA reagent B (sufficient for 3 specimens).
5. During the 15-minute incubation of the TGA plasma and the reagent A + B mixture, prepare 8 labelled microcentrifuge tubes (SPL1–SPL8) for each specimen (test material, reference material or negative control), each containing 490 µL of Buffer B, and place them on ice.
6. At t = 15 min, add 150 µL of the reagent A + B mixture to the tube with plasma and mix gently.
7. At t = 16 min (1 minute after addition of the reagent), transfer a 10 µL sample of the reaction mixture into the tube labelled SPL1, which already contains 490 µL of Buffer B; vortex and place on ice. Mix the reaction tube gently just before taking each 10 µL sample and return it to the water bath immediately after sampling.
8. Repeat this sampling every minute from t = 17 min to t = 23 min to collect SPL2 to SPL8, respectively.
9. Keep all diluted samples on ice until all test materials, reference materials and controls have been processed. Samples can be kept on ice for up to 2 hours. For each additional material or control, start again at step 2.
10. Remove all calibrators and diluted samples in Buffer B from the ice, vortex them and leave them at room temperature.
11. Transfer 150 µL of each calibrator and diluted sample to the 96-well microplate. Table 2 shows a template that may be used.
12. Incubate the plate for 2 minutes at 37 °C.
13. Add 50 µL of Substrate solution to each well. Use of a repeater pipette is highly recommended; pipette in the same order as was used to fill the plate.
14. Cover the plate with a plate cover or sealing film and incubate for 20 minutes at 37 °C.
15. After incubation, add 50 µL of Stop solution to each well, in the same order as the Substrate solution was added (use of a repeater pipette is highly recommended).
16. Read the optical density at 405 nm (OD_{405}) and 540 nm (OD_{540})

within 30 minutes of adding the Stop solution.

17. Calculate the thrombin concentration of all diluted samples and the rate of thrombin generation for each test material, reference material and negative control (see Calculations).

Table 2. Suggested 96-well plate layout for the TGA. CTRL: negative control; REF: reference material; TST: test material; SPL1–8: samples taken at 1–8 minutes.

	1	2	3	4	5	6	7	8	9	10	11	12
A	BLK	CTRL SPL1	REF1 SPL1	REF2 SPL1	TST1 SPL1	TST2 SPL1	TST3 SPL1	TST4 SPL1	TST5 SPL1	TST6 SPL1	TST7 SPL1	TST8 SPL1
B	CAL 1	CTRL SPL2	REF1 SPL2	REF2 SPL2	TST1 SPL2	TST2 SPL2	TST3 SPL2	TST4 SPL2	TST5 SPL2	TST6 SPL2	TST7 SPL2	TST8 SPL2
C	CAL 2	CTRL SPL3	REF1 SPL3	REF2 SPL3	TST1 SPL3	TST2 SPL3	TST3 SPL3	TST4 SPL3	TST5 SPL3	TST6 SPL3	TST7 SPL3	TST8 SPL3
D	CAL 3	CTRL SPL4	REF1 SPL4	REF2 SPL4	TST1 SPL4	TST2 SPL4	TST3 SPL4	TST4 SPL4	TST5 SPL4	TST6 SPL4	TST7 SPL4	TST8 SPL4
E	CAL 4	CTRL SPL5	REF1 SPL5	REF2 SPL5	TST1 SPL5	TST2 SPL5	TST3 SPL5	TST4 SPL5	TST5 SPL5	TST6 SPL5	TST7 SPL5	TST8 SPL5
F	CAL 5	CTRL SPL6	REF1 SPL6	REF2 SPL6	TST1 SPL6	TST2 SPL6	TST3 SPL6	TST4 SPL6	TST5 SPL6	TST6 SPL6	TST7 SPL6	TST8 SPL6
G	CAL 6	CTRL SPL7	REF1 SPL7	REF2 SPL7	TST1 SPL7	TST2 SPL7	TST3 SPL7	TST4 SPL7	TST5 SPL7	TST6 SPL7	TST7 SPL7	TST8 SPL7
H	CAL 7	CTRL SPL8	REF1 SPL8	REF2 SPL8	TST1 SPL8	TST2 SPL8	TST3 SPL8	TST4 SPL8	TST5 SPL8	TST6 SPL8	TST7 SPL8	TST8 SPL8

Calculations*

1. Plot the OD_{405} (corrected for OD_{540}) against the thrombin concentrations of the calibrators. The calibration curve must be a straight line ($OD_{405} = A \times [\text{thrombin}] + B$).
2. Interpolate the thrombin concentrations of all samples at all sampling times.
3. For each test material, reference material and control, construct a thrombin generation curve by plotting the thrombin concentration (mU/mL) against the incubation time.
4. For all test and reference materials, correct the thrombin concentration at each time point for the corresponding thrombin concentration of the negative control(s).
5. For the test materials, the reference materials and the negative control, determine the highest rate of thrombin generation (ΔmU/mL/min) between two consecutive time points (i.e. the steepest part of the curve) and multiply this value by the dilution factor (50).
6. Express the thrombin generation of the test and reference

materials per unit of surface area (ΔmU/mL/min/cm^2). Reference 1 has a surface area of 0.6 cm^2, Reference 2 of 0.7 cm^2 and Reference 3 of 0.6 cm^2.

***An electronic file for performing the calculations is available on request.**

Assay Criteria

- The correlation coefficient of the calibration curve should be ≥ 0.98.
- The parameters A and B of the calibration curve must be within the ranges given in the Certificate of Analysis (enclosed with the kit).
- The result of the negative control should be ≤ 2500 ΔmU/mL/min.
- The results of the reference materials should be within the ranges given in the Certificate of Analysis.
- Repeat the TGA if the results do not meet the assay criteria.

Example Results

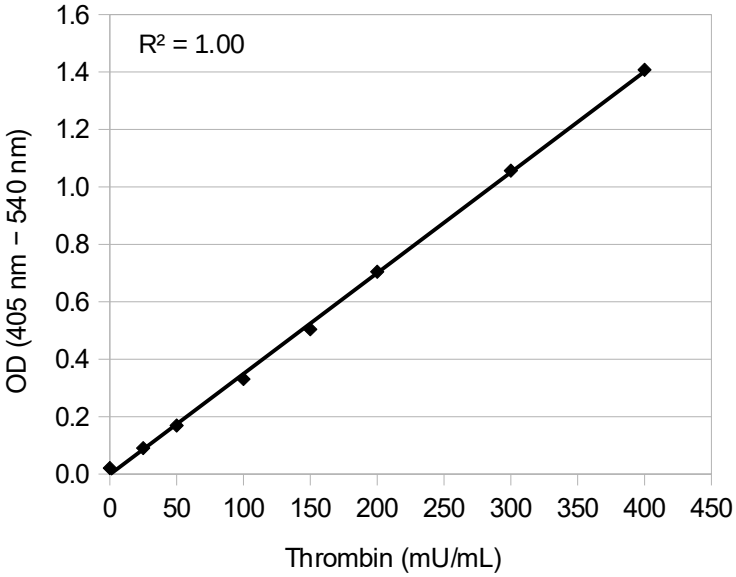

Figure 1. Example of a thrombin calibration curve. This curve is an example only and must not be used for calculations.

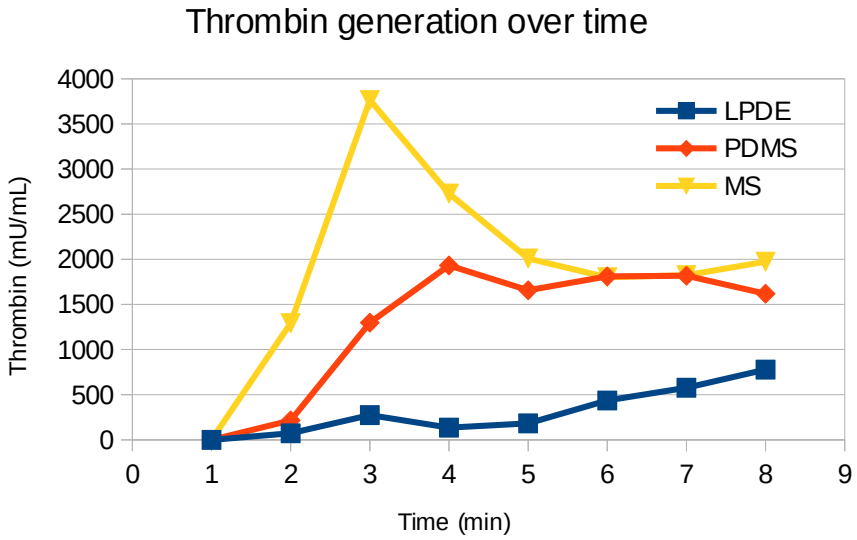

Figure 2. Example thrombin generation curves for low-density polyethylene (LDPE), polydimethylsiloxane (PDMS) and medical-grade steel (MS).

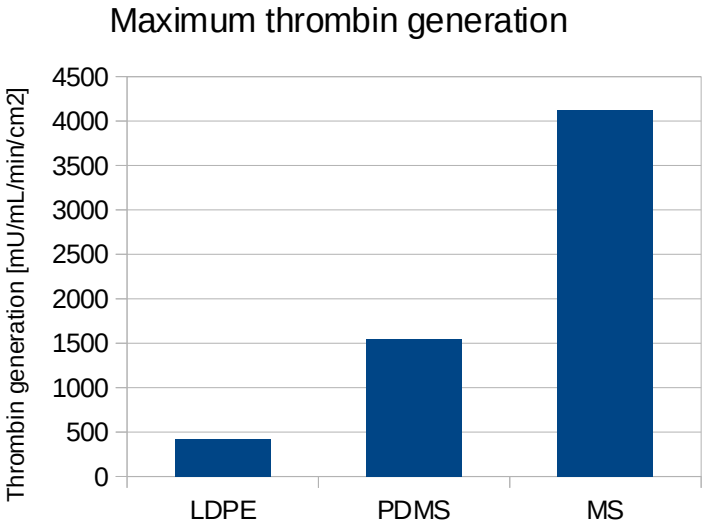

Figure 3. Example maximum rates of thrombin generation for LDPE, PDMS and MS.