Why measure thrombin generation?

How does measurement of thrombin generation work?

Sample material / pre-analytics

Test performance

Applications
Theory of coagulation, 1905

Morawitz, Fuld and Spiro 1905
Coagulation today
Why measure thrombin generation?

The status of haemostasis can be measured in vivo with help of thrombin generation. This includes:

- **plasmatic coagulation**
  - the endogenous system
  - the exogenous system

- **cellular coagulation**
  - influence of platelets
  - influence of microparticles

- **formation of cross linked fibrin**
Scheme of plasmatic coagulation

**Intrinsic pathway**
- Non-endothelial surface
  - F XII → F XIIa
  - F XI → F XIa
  - F IX → F IXa
  - F VIII → F VIIIa
  - F X → F IXa
  - F VIIa → F VII
  - F II → F IIa

**Extrinsic pathway**
- Vascular injury, TF-release
  - TF → F VII → F VIIa
  - F X → F VIII
  - F Va → F V

**TG initiation**
- TG is initiated via the extrinsic pathway.

**Fibrin formation**
- F Xa → F IIa
- Thrombin
- Fibrin monomer
- Fibrin polymer (stable)
- Fibrinogen
The trigger forms a small amount of initial thrombin,

this leads to formation of fibrin

It is rapidly inactivated in a TF/FVIIa/FXa complex by TFPI

Activates by positive feedback the intrinsic system. This means, via factor XI, IX and VII more FXa and thrombin are built.

When „thrombin burst“ gets too big, differences in e.g. FVIII or FIX can’t be measured any more.
Screening tests for plasmatic coagulation
Trigger RD also activated via FXII, XI, IX and VIII - the intrinsic pathway.

Trigger RB, RCL is also activated via the intrinsic pathway due the positive feedback activation of FVIII and FIX.

Trigger RA, RB, RCL, RCH is activated via the extrinsic pathway. Crucial difference is the trigger concentration.

Method dependent continuous detection of formed thrombin
### Choice of suitable trigger for simulating physiological in vivo situations

<table>
<thead>
<tr>
<th>Trigger</th>
<th>Corresponding „in vivo“ situation</th>
</tr>
</thead>
<tbody>
<tr>
<td>no Trigger</td>
<td>no trigger, only endogenous TF and PL in plasma (e.g. microparticles)</td>
</tr>
<tr>
<td>Low PL No TF</td>
<td>resting platelets, no relevant cell activation and/or tissue damage</td>
</tr>
<tr>
<td>Low PL low TF</td>
<td>small vessel damage, without relevant platelet activation and minimal cell activation</td>
</tr>
<tr>
<td>Low PL High TF</td>
<td>Increased TF due to cell activation or tissue damage</td>
</tr>
<tr>
<td>High PL No TF</td>
<td>platelet activation, lipemia</td>
</tr>
<tr>
<td>High PL High TF</td>
<td>increased PL due to platelet activation, increased TF due to cell activation and/or tissue damage</td>
</tr>
</tbody>
</table>
Pattern of the cellular haemostasis

Pattern of the cellular haemostasis

Selection of trigger and method of detection which includes cellular coagulation in measurement

- measurement also in PRP – only possible with fluorogenic method
- Suitable trigger for Microparticle measurement – low PL and TF, only reagent RClow or RB

Chromogenic method only PPP – therefore no detection of microparticles

The structure of fibrin is dependent on thrombin concentration

Blombäck et al. (1994) Thromb Res 75: 521-538
Generated amount of thrombin is dependent on formation of the fibrinnet. By addition of INH the fibrin net can’t be formed.

- Peak Thrombin one third lower

Choice of detection method which allows formation of the fibrinnet

- no addition of clot inhibitor – only in fluorescence measurement possible
**Control of thrombin formation**


**TECHNOTHROMBIN® TGA**
Thrombin Generation Assay

Difference to classical methods influence of inhibitors during the whole measurement
Physiological balance of coagulation

FI fibrinogen-fibrin
FII prothrombin-thrombin
FIII tissue factor TF
FIV Ca+
FV/FVI/FVII/FVIII/FIX/FXI/FXIII

AT antithrombin
PC Protein C
PS Protein S
TFPI tissue factor pathway inhibitor
Thrombomodulin

Balance between coagulation factors (C) and inhibitors (I)

Deficiency of factors
Risk of bleeding

Deficiency of inhibitors
Risk of thrombosis
How does measurement of thrombin generation work?

The status of haemostasis can be measured in vivo with help of thrombin generation.
The coagulation cascade is activated by addition of a TRIGGER.

The formed thrombin is cleaved by an external substrate and the signal is continuously recorded.

Alternatively generation of fibrin is recorded.
Currently there are three commercially available methods for measurement of thrombin generation.
Coagulation cascade is activated by addition of different concentrations of
- Tissue factor and
- phospholipids

Thrombin generation is based on measurement of changes in fluorescence, which are caused by cleavage of a fluorogenic substrate by thrombin
Why TECHNOTHROMBIN® TGA does not correct for α2MG-Thrombin complex

"in vivo" the complex α2MG-Thrombin is inactive, because Fibrinogen, the natural substrate for Thrombin has no access to the active center.

Small substrates such as the fluorogenic substrate ZGGR-AMC are cleaved by α2MG-Thrombin.
Why TECHNOTHROMBIN® TGA does not correct for α2MG- Thrombin complex

α2-MG Thrombin can be corrected mathematically

but:

- Peak Thrombin is not influenced significantly by α2-MG
- α2-MG concentrations can vary significantly in newborn, children, and different patient groups, so that a mathematical correction can rise the measurement error (Ignatovic, BJH 138(3), 2007)
Thrombin generation tests detect the whole kinetic of Thrombin generation

- lag phase
- peak time
- slope / velocity index

\[
\text{velocity index} = \frac{\text{peak thrombin}}{\text{peak time} - \text{lag phase}}
\]

- peak thrombin
- area under the curve (AUC)

Using conventional coagulation tests only detect the initial phase of thrombin generation with endpoint “generation of first fibrin”
When thrombin concentration in function of time is plotted, a thrombin generation curve is obtained, which shows different phases of thrombin generation.
Sample material / pre-analytics
Sample material

- platelet rich plasma (PRP)
- platelet poor plasma (PPP)
- platelet- and microparticle free plasma (PFP) can be used

Preparation of:

Platelet rich plasma (PRP):
Centrifuge 5 minutes at 100 x g and carefully pipette off the obtained PRP.

Platelet poor Plasma (PPP):
- Centrifuge PRP 10 minutes at 1.500 x g and carefully pipette off the obtained PPP.
- Alternative: Centrifuge whole blood 15 minutes at least 2500 x g (according to norm DIN 58905).

Platelet- and microparticle free Plasma (PFP):
- Centrifuge PPP 30 minutes at least at 15.000 x g and carefully pipette of the obtained PFP.
- Or by 2 minutes of filtration via Ceveron® MFU 500.
Sample material

A blood sample can be collected with:
- Citrated blood collection tubes
- CTAD tubes
Mechanical agitation (sample transport by letter shoot) can lead to significant changes in thrombin generation.

Influence of sample transport on thrombin generation in 3 healthy donors (TECHNOTHROMBIN® TGA, RB Reagent).

Samples should be transported only after centrifugation!

L. Wiens, Magdeburg Poster 140 GTH 2006
Sample material

We recommend:

- CTAD tubes for blood collection
- Samples should be centrifuged right after collection
- Samples should only be transported after centrifugation
- Plasma samples which need to be stored should be frozen immediately after centrifugation
- Frozen samples should be stored at constant temperature – avoid temperature variations during storage.
Test performance
Reagent preparation

We recommend:

- The lyophilized reagents must be dissolved in the volume of distilled water indicated on the vials.
  - After exactly **20 minutes of reconstitution time** and thorough mixing (Vortex), controls, calibrator and substrate are ready to use.
  - The trigger reagents (RA, RB, RClow, RChigh and RD) have a reconstitution time of exactly 20 minutes and should be used immediately afterwards.
  - The reagent mixture (trigger reagent + substrate) is made after the recommended reconstitution time for the trigger of 20 minutes and should be used – like all reagents – immediately afterwards.
  - All reconstituted reagents including the aqua dest should reach room temperature before usage.
The calibration curve (thrombin curve) enables conversion of results from RFU/min to nM thrombin.

The calibration curve is created **separately from sample measurement**.

For each **lot of substrate only one calibration curve** has to be created.

**CALIBRATION CURVE (CAL)**

40 µL dilution of calibrator (CAL)
50 µL TGA substrate (SUB)

Measurement period: **10 min**
Filters: ~360 nm / ~460 nm
Measurement interval: **30 sec**

4 different dilutions of thrombin calibrator
- **Sample measurement** is performed *separately from calibration curve*.

- As an alternative, a reagent/substrate mixture can be prepared in advance to reduce pipetting steps.

**SAMPLES**

- 40 µL sample
- 10 µL reagent (**RA, RB, RC low, RC high***)
- 50 µL substrate (SUB)

measurement period: **60 / 120 min**

Filters: ~ 360 nm / ~ 460 nm

measurement interval: **1 min**

* **ATTENTION:** Different pipetting scheme for **RD** see next page
Sample measurement is performed separately from calibration curve.

As an alternative, a reagent/substrate mixture can be prepared in advance to reduce pipetting steps.

**SAMPLES**

- 20 µL sample
- 30 µL reagent (RD)
- 50 µL substrate (SUB)

measurement period: **60 / 120 min**

Filters: ~ 360 nm / ~ 460 nm

measurement interval: **1 min**
We recommend following reagents for the determination of:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGA RA</td>
<td>- to monitor the activity of microparticles</td>
</tr>
<tr>
<td>TGA RB and RC Low</td>
<td>- Measurement of thrombophilic tendency (preferentially with platelet poor plasma PPP)</td>
</tr>
<tr>
<td></td>
<td>- Measurement of bleeding tendency</td>
</tr>
<tr>
<td></td>
<td>- For monitoring FVIII inhibitor Bypass therapy with rFVIIa and FEIBA</td>
</tr>
<tr>
<td></td>
<td>- hF VII, hF Xa, hF XIa</td>
</tr>
<tr>
<td></td>
<td>- to monitor the thrombogenity of microparticles</td>
</tr>
<tr>
<td>TGA RC High</td>
<td>- for monitoring of anticoagulant therapy</td>
</tr>
<tr>
<td>TGA RD</td>
<td>- For monitoring heparin, direct thrombin and Xa inhibitor therapy</td>
</tr>
<tr>
<td></td>
<td>- hF XIIa, plasma callicrein, callicrein1 (Tissue Factor)</td>
</tr>
</tbody>
</table>
The concentration of the different TGA reagents are:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGA RA</td>
<td>Low conc. of phospholipid micelles containing <em>no</em> rhTF Tris-Hepes-NaCl buffer</td>
</tr>
<tr>
<td>TGA RB</td>
<td>Low conc. of phospholipid micelles containing <em>low</em> rhTF in Tris-Hepes-NaCl buffer</td>
</tr>
<tr>
<td>TGA RC Low</td>
<td>Low conc. of phospholipid micelles (same as in RB) containing <em>high</em> rhTF in Tris-Hepes-NaCl buffer</td>
</tr>
<tr>
<td>TGA RC High</td>
<td>High conc. of phospholipid micelles containing <em>high</em> rhTF (same as in RCL) in Tris-Hepes-NaCl buffer</td>
</tr>
<tr>
<td>TGA RD</td>
<td>Special composition of phospholipids</td>
</tr>
</tbody>
</table>
For determination of thrombin generation a fluorescence reader, which is equipped with filters of wavelength ~360/~460 (excitation/emission) is needed.

- **Fluorescence reader** with Excel evaluation software

Or

- **Ceveron® alpha** fully automated coagulation analyzer
AVAILABLE READER APPLICATIONS

✓ BioTek® FLx 800™ TBI (Software Gen 5/ KC 4 / KC Junior)
✓ BMG Labtech FLUOstar OPTIMA
✓ Molecular Devices Gemini / SpectraMax
✓ Perkin Elmer ® Victor Wallac
✓ TECAN Genios / Infinite
✓ Thermo Fluoroskan

ATTENTION: For accurate results we recommend to change the lamp of your reader every year.
Evaluation software example

Plate Layout

Calibration Curve

Results
Evaluation software download
www.technoclon.com
TECHNOTHROMBIN® TGA
Thrombin Generation Assay

- Reader Settings
- Reader protocols (for corresponding reader software)
- Evaluation software
- Software description
Ceveron® alpha

FULLY AUTOMATED COAGULATION ANALYZER
For routine, research and new generation tests!

clotting
TPT, aPTT, TT, Fibrinogen, ...
II, V, VII, IX, X, XI, XII, XIII; ...
Protein C, Lupus, APC, ...

chromogenic
AT III, Protein C, C1 INH,
FVIII:C, ...

turbidimetric
Lp(a), D-Dimer, CRP, ...

fluorometric
Thrombin Generation
For thrombin generation measurement a special fluorometric TGA module is placed over the cuvette rotor with a UV emitter (365nm)
Ceveron® alpha

TECHNOTHROMBIN® TGA
Thrombin Generation Assay

TGA monitoring of each individual sample over measurement period possible
Results given for:
Lag time, Peak time, Peak, VI; AUC
Applications
Goal

- Measurement of an individual coagulation potential in relation to a phenotypic diagnostic.
- Correlation of bleeding events
- Detection of hypercoagulability
- Measurement of the effect of anticoagulant drugs (independent of the class of medication)
Hemophilia

- Differentiating the grade of hemophilia (Santagostino et al. Haemophilia 2005)
- Improved monitoring of substitution therapy
- Monitoring of therapy with bypassing-concentrates (Varadi et al. Haemophilia 2004)
Hemophilia

Thrombin generation in hemophilia A after infusion of 50IU/kg FVIII

Barrowcliffe; Haemophilia 2006, 12(Suppl.3)
Thrombophilia

- Identification of thrombotic risk factors
- Factor II mutation
Thrombophilia

AUREC STUDY / TECHNOTHROMBIN TGA RC LOW

• N=914
  – First idiopathic venous thromboembolism
  – Prospective cohort study
  – Observation 47 month after ending of oAc.

• N=100 patients with VTE-recurrence (11%)
  – Thrombin generation > 400nM probability of recurrence 20%
  – Thrombin generation < 400 nM 6,5% after 4 years

Hron G. et al., JAMA, July 26, 2006 – Vol 296, No. 4, 397-402
Patients can be stratified according to their risk of recurrence by a simple global coagulation assay

Low risk patients represent 2/3 of patients

no need for anticoagulants

lower risk of bleeding

Hron G. et al., JAMA, July 26, 2006 – Vol 296, No. 4, 397-402
Figures 1, 2 and 3 clearly show that thrombin generation significantly increases during pregnancy. This increase can be monitored with all three reagents which differ in their concentrations of tissue factor and phospholipid concentration. In contrast to the classical activation markers – Prothrombin fragment 1+2 and D-Dimer (Fig. 4) – there is no significant difference in thrombin generation between 2nd and 3rd trimester.
Anticoagulation therapy

Fondaparinux and Enoxaparin

Thrombin generation in PRP

Influence of fondaparinux

Influence of enoxaparin

Effect of increasing concentrations of fondaparinux (left panel) and enoxaparin (right panel) on thrombin generation in platelet-rich plasma (PRP) after tissue factor pathway activation. Representative ‘thrombograms’ of one out of seven experiments. a, control; b, 0.11 anti-FXa IU/ml; c, 0.28 anti-FXa IU/ml; d, 0.57 anti-FXa IU/ml; e, 0.91 anti-FXa IU/ml; f, 1.14 anti-FXa IU/ml of fondaparinux. A, control; B, 0.1 anti-FXa IU/ml; C, 0.25 anti-FXa IU/ml; D, 0.5 anti-FXa IU/ml; E, 0.8 anti-FXa IU/ml; F, 1 anti-FXa IU/ml enoxaparin.
Anticoagulation therapy

Hirudin

TECHNOTHROMBIN® TGA
Thrombin Generation Assay

A. Siegemund, Leipzig
Anticoagulation therapy

TECHNOTHROMBIN® TGA
Thrombin Generation Assay

Melagatran

Control
0.2 µM
0.4 µM
0.6 µM
0.8 µM

Thrombin (nM)

Time (min)

A. Siegemund, Leipzig
Microparticles

- Plasma microparticles are spherical cell membrane fragments derived from apoptotic or activated cells.
- They are rich in phospholipids and proteins, e.g. tissue factor, and thus are thrombogenic.
- Microparticles are thought to be one of the major risk factors for thrombosis in atherosclerotic patients.
- Determination thrombin generation by microparticles would allow to directly relate their circulating levels to the micro particle-induced thrombotic tendency.
How do we use the thrombin potential?

- Risc stratification after venous thromboembolism
- Thrombophilia
  - Monitoring of anticoagulant therapy?
  - pregnancy?
- hemophilia
  - Individually monitoring of therapy
  - Until now no diagnostic benefit for bleeding tendency