Application Note

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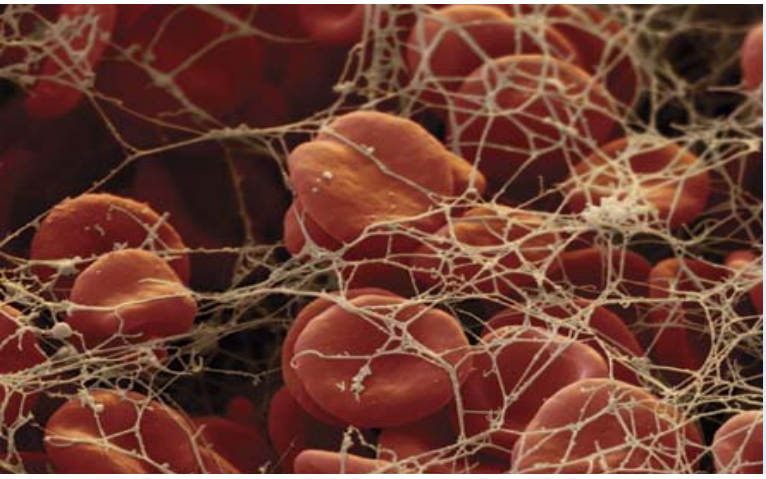
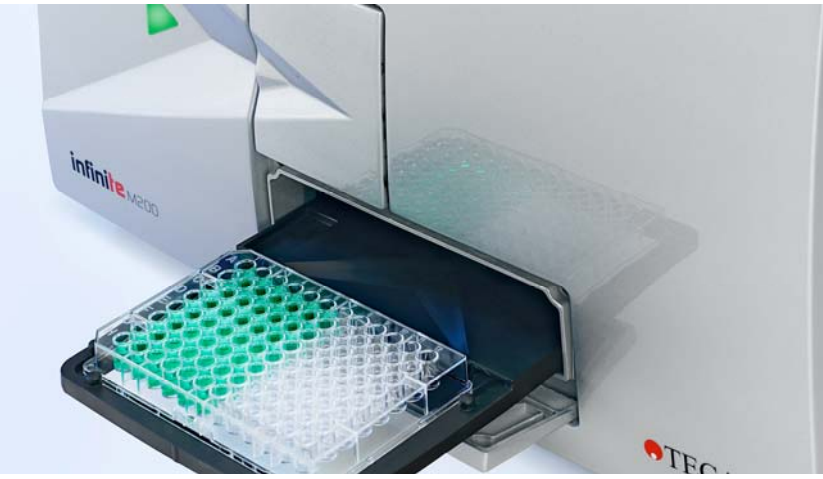
TECAN

Technothrombin® TGA Assay on Tecan´s Infinite® M200

Fluorescence Intensity based Monitoring of Thrombin Generation





Introduction

Thrombin is a protein playing an important role in the coagulation cascade (Figure 1). It is a serine protease that converts soluble fibrinogen into insoluble strands of fibrin which strengthen the platelet plug. Disorders of coagulation lead e.g. to hemorrhage or thrombosis.

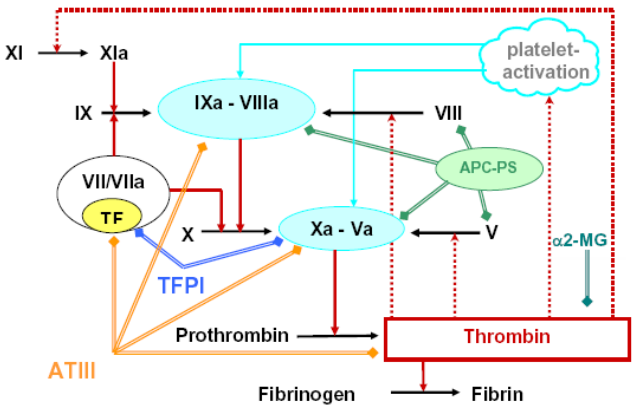


Figure 1: Scheme of the coagulation cascade [1].

Technothrombin TGA

Assay principle

The Technothrombin® TGA assay from Technoclone, Austria monitors the formation of thrombin over time by means of a converting a fluorescent substrate upon activation of the coagulation cascade by tissue factor (extrinsic pathway) and negatively charged phospholipids in blood plasma. It allows measuring the whole kinetics during initiation, amplification and down-regulation of thrombin formation. Changes in fluorescence are converted to thrombin concentrations using the thrombin calibration curve [2].

Experiment

The influence of micro particles on thrombin generation in human blood plasma was investigated using the Technothrombin® TGA Kit and Tecan´s Infinite M200 multimode microplate reader.
Plasma with (PPP = platelet poor plasma) and without micro particles (PFP = particle free plasma) from the same origin was prepared and the thrombin generation was monitored by measuring increasing fluorescencne signals over time. Results were evaluated using the Technothrombin® TGA evaluation software and compared between PPP and PFP.

All experiments were performed at the Division of Haematology, University Childrens's Hospital Zurich by Oliver Speer, PhD using the Tecan Infinite® M200 instrument.

Materials and Methods

Instruments

- Tecan Infinite® M200 multimode microplate reader

Assay

- Technothrombin® TGA Kit (Technoclone, Austria)

Microplate

- 96 well microplate, MaxiSorp, black, flat bottom (Nunc, Denmark

Evaluation

- Technothrombin® TGA evaluation software (Technoclone, Austria)

Thrombin calibrators
The preparation of the calibrators was done according to the instructions of the manufacturer [2]. The measurement is started immediately after adding the substrate. All calibrator dilutions were measured in duplicate. The measurement data was analyzed with the Technothrombin® TGA evaluation software. Only one calibration curve has to be done for each lot of the assay.

Measurement parameters and settings are given in Table 1. The Infinite M200 instrument was preheated to 37°C at least half an hour before starting to measure.

Measurement Parameter	Instrument Settings
Plate	NUN96fb
Kinetic	
Shaking (Orbital) Duration	5 s
Shaking (Orbital) Amplitude	4 mm
Wait (Time)	3 s
Fluorescence	
Kinetic Cycles	21
Interval Time	30 s
Mode	Fluorescence Intensity Top
Excitation wavelength	360 nm
Excitation bandwidth	9 nm
Emission wavelength	460 nm
Emission bandwidth	20 nm
Gain manual	50
Number of Flashes	12
Integration Time	20 µs
Lag Time	0 µs
Settle Time	0 ms

Table 1: Measurement parameter and instrument settings on Infinite M200 for calibrators.

Blood plasma samples
Citrated blood from healthy children (age 2 - 14) was centrifuged 10 min at RT at 2200 g. One half of the supernatant, platelet poor plasma (PPP) was aliquoted and frozen at –20°C, the other half was centrifuged 30 min at 4°C at 13000 g. The resulting supernatant, particle free plasma (PFP) was aliquoted and stored at -20°C.

The thrombin generation assay was performed with TGA RC low (low concentration of phospholipids micelles) according to the manufactures instructions [2]. As controls the following samples were added: Normal human plasma, human plasma with increased thrombin generation and human plasma with decreased thrombin generation.

The measurement is started immediately after adding the substrate. The measurement settings for the samples are given in Table 2. The Infinite M200 instrument was preheated to 37°C at least half an hour before starting measurements.

Measurement Parameter	Instrument Settings
Plate	NUN96fb
Kinetic	
Shaking (Orbital) Duration	5 s
Shaking (Orbital) Amplitude	4 mm
Wait (Time)	3 s
Fluorescence	
Kinetic Cycles	61
Interval Time	1 min
Mode	Fluorescence Intensity Top
Excitation wavelength	360 nm
Excitation bandwidth	9 nm
Emission wavelength	460 nm
Emission bandwidth	20 nm
Gain manual	50
Number of Flashes	12
Integration Time	20 µs
Lag Time	0 µs
Settle Time	0 ms

Table 2: Measurement parameter and instrument settings on Infinite M200 for samples.

Calculations
All calculations were done with the Technothrombin® TGA evaluation software. Measured RFU data of the calibrators were converted to the thrombin calibration curve which was then used to calculate generated thrombin [nM] present in the samples at a given time. The software calculated the Thrombin formation graph automatically and provided the following key data: lag-phase, peak-thrombin, peak-time, Velocity-Index (highest rate of thrombin formation per minute), and Area under Curve.

Results

All shown data were provided by Oliver Speer PhD, University Childrens's Hospital Zurich using Tecan's Infinite M200.

Thrombin calibration curve

The raw data of calibrators are shown in Figure 2. These data were imported into the Technothrombin® TGA evaluation software to calculate the Thrombin calibration curve (Figure 3).

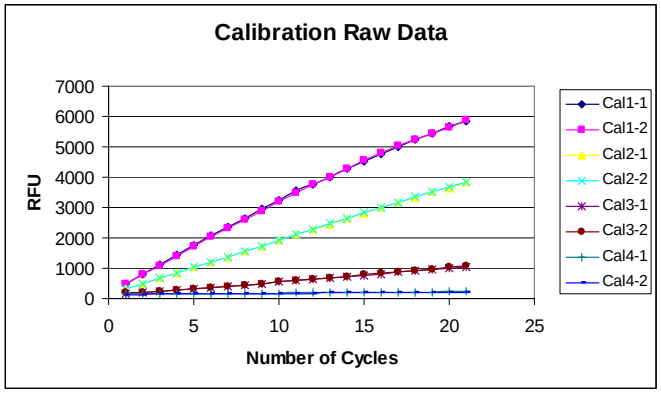


Figure 2: Measured raw data of calibrators using the Infinite M200.

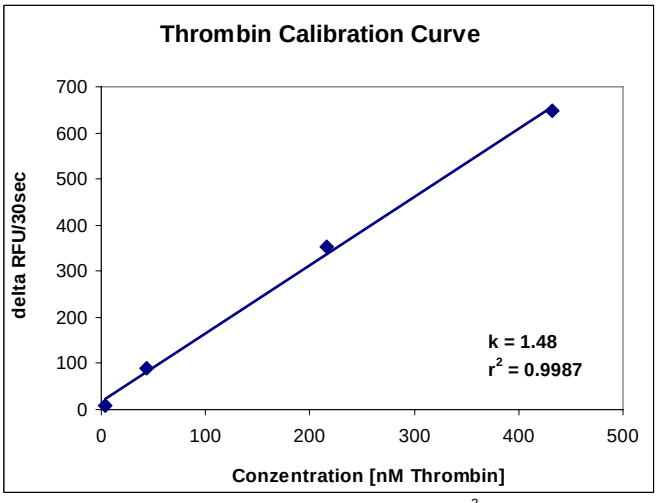


Figure 3: Thrombin Calibration Curve: k = slope; r^2 = regression.

Plasma samples

Measurement raw data was imported into the Technothrombin® TGA evaluation software to calculate key data of Thrombin formation. A summary of results is given in table 3.

Thrombin generation in PPP was about two times higher than thrombin generation found in PFP. The maximum concentration of Thrombin was 305.7±64.9 nM in PPP Thrombin and 138.1±44.2 nM in PFP (Figure 4). The maximum of thrombin formation in the PPP occurred about 18±3.6 minutes after starting the reaction by adding the substrate contrary to PFP where it takes about 32±10.9 minutes on average.

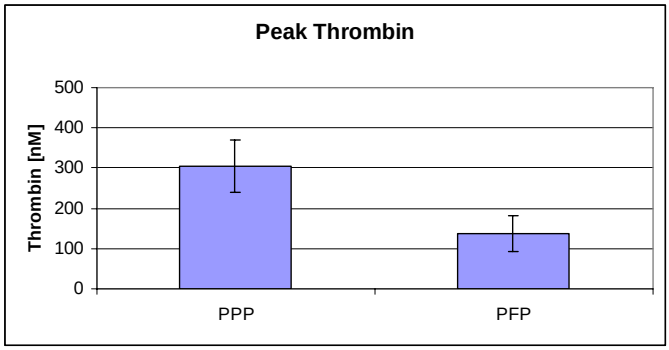


Figure 4: Peak Thrombin in PPP and PFP.

Table 3: Summary of measurement results of human plasma samples.

Sample No.		1	2	3	4	5	6	7	8	AV	SD
Plasma Treatment		Platelet Poor Plasma (PPP)									
Lag Phase	Time [min]	10,5	3,5	10,8	9,8	14,5	3,0	4,0	16,6	9,1	5,2
Peak Height	Thrombin [nM]	323,0	218,9	310,1	366,9	243,3	241,2	345,7	396,2	305,7	64,9
	Time [min]	16,5	17,2	17,4	14,4	22,0	22,1	13,1	22,1	18,1	3,6
Velocity-Index		55,3	16,0	47,5	81,7	33,1	12,6	38,3	72,8	44,7	24,8
Area under Curve		3851	5131	4501	4125	4371	5371	6125	3729	4650,4	827,1
Sample No.		1	2	3	4	5	6	7	8	AV	SD
Plasma Treatment		Particle Free Plasma (PFP)									
Lag Phase	Time [min]	20,5	23,7	14,4	14,4	16,0	23,6	19,6	47,1	22,4	10,7
Peak Height	Thrombin [nM]	180,3	88,1	173,8	197,8	111,9	80,3	120,4	152,1	138,1	44,2
	Time [min]	28,0	36,3	23,9	19,9	24,0	37,6	30,6	54,1	31,8	10,9
Velocity-Index		24,1	7,0	18,2	36,1	14,1	6,4	14,7	22,1	17,8	9,7
Area under Curve		3117	2068	3849	3272	2820	1763	2520	1374	2597,9	829,8

Conclusion

The amount of generated thrombin is dependent on the number of micro particles present in the sample. PFP showed a delayed and lower Thrombin formation compared to PPP.

This application note describes the successful implementation of the fluorescence intensity based Technothrombin® TGA assay on Tecan´s Infinite M200 monochromator based multimode detection system.

Acknowledgements

We express our acknowledgements to Oliver Speer, PhD from the Division of Haematology, University Childrens's Hospital Zurich who provided the data and Veronika Binder and Viktoria Kaufmann from Technoclone GmbH (Vienna, Austria) for their technical support.

List of abbreviations

TGA	thrombin generation assay
PPP	platelet poor plasma
PFP	particle free plasma
RFU	relative fluorescence unit
AV	average
SD	standard deviation

Literature

[1] Technoclone homepage, www.technoclone.com
[2] Technothrombin TGA Product Insert

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