Assessing thrombin generation using the TECHNOTHROMBIN TGA kit and SpectraMax i3x Multi-Mode Microplate Reader

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Introduction

Assessing the generation of thrombin in a plasma sample allows researchers to better understand coagulation mechanisms. To accommodate the needs of researchers to determine the time-dependent changes in thrombin concentrations on a flexible platform, Technoclone has developed a plate reader-compatible kinetic assay format, the TECHNOTHROMBIN® TGA (thrombin generation assay) kit, using a fluorogenic substrate. Cleavage of the substrate occurs upon activation of the coagulation cascade by different concentrations of tissue factor and negatively charged phospholipids in plasma. Researchers can choose from a range of three triggers with the kit to study different areas of coagulation mechanisms. These areas include thrombophilia, haemophilia, anticoagulation, thrombogenicity of microparticles, and drug development studies. In addition, TGA has been proposed to evaluate the thrombogenic activity of immunoglobulin concentrates. All three triggers were tested on the SpectraMax® i3x Multi-Mode Microplate Reader with excellent results.

- TECHNOTHROMBIN TGA RB Trigger composition is specially adapted to detect very sensitive changes in the low range of FVIII and FIX levels in bleeding tendency and for monitoring therapy in heamophilia patients.
- TECHNOTHROMBIN TGA RC low Trigger composition is specially adapted for measurement of thrombophilic tendency, anticoagulant therapy with DOACs and to detect thrombogenic of microparticles.
- TECHNOTHROMBIN TGA RC High trigger composition is specially adapted to induce a thrombin burst upon activation of the extrinsic pathway, which allows the measurement of thrombin generation in samples anticoagulated with Heparin, LMWH or Warfarin.

Instrument and Reagents

- SpectraMax i3x Multi-Mode Microplate Reader (Molecular Devices) with FI-COFL Detection Cartridge (Molecular Devices, PN# 0200-7002)
- FluoroNunc™ Framed Well Modules black (Thermo Scientific, PN# 475515)
- TECHNOTHROMBIN TGA Kit with 3 different triggers, as well as Calibrator and Control sample (Technoclone, PN# 5006010)

Methods

Data Acquisition

The plate reader acquisition protocol used for both the thrombin calibration curve as well as the sample measurement was prepared according to the application report from Technoclone, which can be found on www.technoclone.com. The protocol is applicable for SoftMax® Pro Software version 6.3 and higher. The instrument settings for the SpectraMax i3x plate reader are summarized in Table 1. The FI-COFL cartridge set-up was chosen, as it has superior performance and increased dynamic range over the monochromator path, but the monochromator path can be used as well (data comparison not shown). The plate reader was set to 37°C and allowed to warm up for at least 30 minutes before the read was started.

The thrombin calibration curve was prepared in duplicate with a thrombin calibrator concentration ranging from 3.6 to 361 nM. Immediately after pipetting of the fluorogenic substrate, the plate was transferred to the plate reader to start the acquisition of the kinetic profile over 10 minutes and 30 seconds, with a read interval of 30 seconds.

The sample plate was then prepared by adding the triggers to a plasma sample. The plate was immediately transferred to the plate reader after adding the fluorogenic substrate and was measured for 1 hour at 1-minute intervals.

Parameter	SpectraMax i3x
Optical Configuration	FI-COFL Detection Cartridge
Read Mode	Fluorescence
Read Type	Kinetic
Wavelengths	EX 360 nm – BW 35 nm EM 465 nm – BW 35 nm
PMT and Optics	Integration Time: 400 ms Read from Top Read Height: 0.8 mm (optimized)
Shake	Before first read: 5 seconds, linear, medium

Table 1. Plate Reader settings for thrombin calibration curve and sample plate.

Methods

Data Analysis: TECHNOTHROMBIN TGA evaluation file

- The raw data for each measurement was exported from SoftMax Pro Software to Excel. Data from www.technoclone.com with customer login), where an optimized algorithm corrects for the inner filter effect caused by fluorescent molecules which absorb the light from other product molecules.
- The raw data obtained from the thrombin calibration curve measurement was used from time point 30 seconds. The readout and measurement of the calibration curve can be used for all further sample measurements using the same lot of TECHNOTHROMBIN TGA substrate. The raw data obtained from the sample measurement were copied into the same evaluation file for analysis.

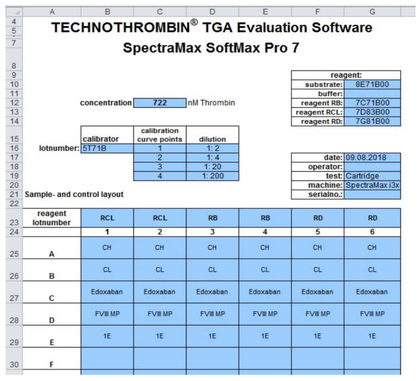

Figure 1. Plate layout with all information for reagent lots, calibrator, operator, instrument, and the plate layout used for sample measurement.

Results

Thrombin Calibration Curve

The raw data of the thrombin calibrators in SoftMax Pro Software are shown in Figure 2. The increase of the signal of the cleaved fluorogenic substrate is relating to the thrombin concentration. For each calibrator, the signal increase is calculated as ΔRFU (RFU/min) and is used to generate the calibration curve in Figure 3. The calibration curve is used to convert ΔRFU of sample measurement to nM Thrombin.

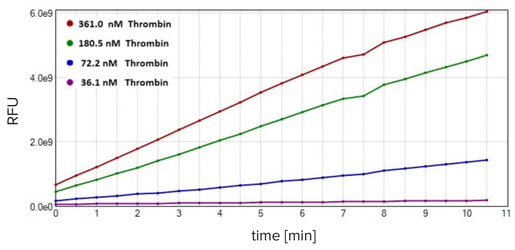

Figure 2. Raw data kinetic plots of thrombin calibrators in SoftMax Pro Software.

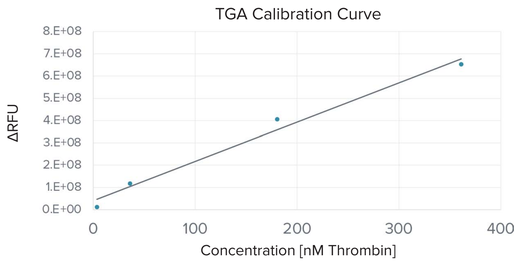

Figure 3. Thrombin calibration curve in TECHNOTHROMBIN TGA evaluation file.

Thrombin Generation Curve

From the changes in fluorescence over time, the sample's concentration of thrombin (nM) can be calculated using the respective thrombin calibration curve. The increase in thrombin concentration with time then allows one to calculate generation of thrombin in the sample and to plot these thrombin values over time for the whole coagulation process. In general, the thrombin generation curve describes the variation of the thrombin amount during the activation of the coagulation cascade and visualization of the different phases of clot formation (Figure 4). To generate the thrombin generation curve, the raw kinetic profiles of the samples (Figure 5) are analyzed in the TECHNOTHROMBIN TGA evaluation file for analysis (Figure 6). First an optimized algorithm calculates the first derivative of sample raw data, and after that the calibration curve is used to further convert the ΔRFU of sample measurement to nM thrombin. The TGA curve is used to calculate all thrombin generation parameters (Table 2).

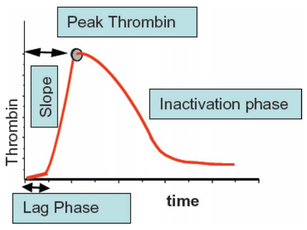

Figure 4. Thrombin generation curve and phases of clot formation.

Results

The calculated parameters as well as the thrombin generation curves reflect the thrombogenic potential of each sample. Results of a normal plasma sample, a thrombophilia patient sample, and a sample from a haemophilia A patient are shown in Table 2. All parameters of the thrombophilia patient sample are increased in comparison to those of the normal sample, indicating its high thrombogenic potential. The parameters of the haemophilia A plasma sample are reduced in comparison to those of the normal sample, reflecting the bleeding tendency of these patient groups caused by a missing or defective factor VIII.

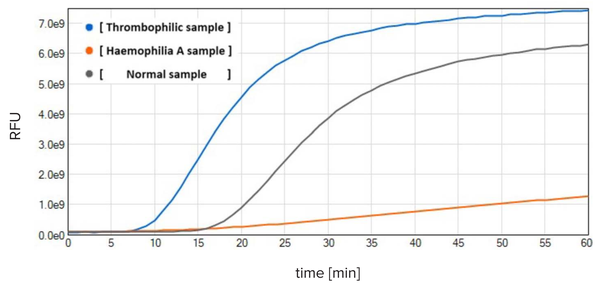

Figure 5. Raw data kinetic plots of sample measurements in SoftMax Pro software triggered with trigger TECHNOTHROMBIN TGA RB.

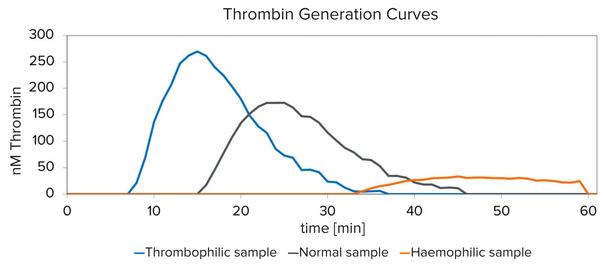

Figure 6. Transformed Thrombin generation curves of a normal plasma sample, a thrombophilia patient sample, and a sample from a haemophilia A patient.

Sample	Trigger Reagent	lag phase time [min]	peak height Thrombin [nM]	peak height time [min]	Slope (Velocity Index)	AUC
Thrombophilic Sample	RB	8	269.7	15	38.53	3331
Normal Sample	RB	14	172.6	25	15.69	2570
Haemophilia A Sample	RB	29	33.6	45	2.10	662

Table 2. Results of a normal plasma sample, a thrombophilia patient sample, and a sample from a haemophilia A patient obtained with the TECHNOTHROMBIN® TGA evaluation file.

Assay Validation Results on SpectraMax i3x

With the standardized TECHNOTHROMBIN TGA reagents and the validated protocols, a precise measurement with CVs < 10% is possible on the SpectraMax i3x reader with a FI-COFL detection cartridge (see Table 3). TECHNOTHROMBIN TGA RC Low was chosen as an example, as this trigger is most commonly used.

	lag phase time [min]	peak height Thrombin [nM]	peak height time [min]	AUC
Target value	7.3	311.4	14.2	3609
Intra-assay CV %	6.2	6.0	5.3	1.7

	lag phase time [min]	peak height Thrombin [nM]	peak height time [min]	AUC
Target value	7.3	274.6	14.3	3312
Inter-assay CV %	6.5	9.8	3.3	5.1

Table 3. Intra-assay CVs and Inter-assay CVs of TGA parameters for trigger RC Low.

Conclusions

The SpectraMax i3x reader, together with SoftMax Pro Software and TECHNOTHROMBIN TGA evaluation file, offers an ideal platform to perform TGA with good precision using modular TECHNOTHROMBIN TGA reagents. With validated settings and standardized reagents, all the requirements for use of thrombin generation in research projects in the field of thrombophilia, haemophilia, anticoagulation, thrombogenity of microparticles, or drug development studies are met.

- For more information on the SpectraMax i3x plate reader, please contact us on our website https://www.moleculardevices.com/contact
- For more information on TECHNOTHROMBIN TGA Kit (Technoclone GmbH) please contact products@technoclone.com

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