CREATIVE BIOMART®
Assay Kit

Thrombin Generation Assay Kit

Product Information

Cat.No. Kit-0982

Product Overview

The TGA kit is an assay system for determination of thrombin generation over time in platelet poor or platelet rich plasma (PPP or PRP) upon activation of the clotting cascade by micelles of negatively charged phospholipids containing different amounts of human tissue factor and CaCl2. The kit can be used to monitor hemophiliacs during inhibitor bypassing therapy, to monitor anticoagulation therapy, to calculate INR values for patients and to determine states of bleeding disorders or thrombophilia as well as the activity of circulating micro particles. This broad range of applications is possible by providing different tissue factor concentrations and by monitoring the whole kinetic of thrombin generation during initiation, amplification and down regulation of thrombin formation. TGA is therefore a universal assay kit for analyzing and monitoring the function of the haemostatic system.

Description

TGA is based on monitoring the fluorescence generated by the cleavage of a fluorogenic substrate by thrombin over time upon activation of the coagulation cascade by different concentrations of tissue factor and negatively charged phospholipids in plasma. From the changes in fluorescence over time, the concentration of thrombin (nM) in the sample can be calculated using the respective thrombin calibration curve. The increase in thrombin concentration with time then allows to calculate generation of thrombin in the sample and to plot such thrombin values over time for the whole coagulation process. This then results in the visualization of the different phases of clot formation.

Storage

Reagent RT*(20...25°C) +2…8°C -20°C
TGA substrate (SUB) 1 week 1 month 6 months
TGA buffer (BUF) 8 hours 1 week 1 month
TGA thrombin calibrator (CAL) 8 hours 1 week 6 months
TGA reagent B (RB), C Low (RCL) , High (RCH), D (RD) 8 hours 1 week 6 months

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TGA control high (CH) 4 hours 8 hours 1 month
TGA control low (CL) 4 hours 8 hours 1 month
Avoid contamination by micro-organisms.
Plasmas should be frozen only once; during storage, the vials should be tightly capped.
Stability of the sample material: * room temperature
Sample material RT* (20...25°C) +2…8°C -20°C
PPP, PRP and PFP Plasma 2 hours 4 hours 1 month
An immediate centrifugation after blood withdrawal is recommended.
Further we recommend an immediate shock freezing of the centrifuged samples.
Attention! The frozen samples should be stored in a constant environment - avoid exposing the samples to variations in temperature.
Before transportation we recommend to centrifuge and prepare the samples.

Size

3x16 tests

Kit Components

Substrate: 3 x 1.5 TGA substrate (SUB) Fluorogenic substrate 1 mM Z-G-G-RAMC, 15 mM CaCl2
Buffer: 1 x 3 TGA buffer (BUF) Hepes-NaCl-buffer containing 0.5 % bovine serum albumin
Calibrator: 1 x 0.5 TGA thrombin calibrator ~1.000 nM thrombin in buffer with BSA
Reagent C Low: 1 x 0.5 TGA reagent C (RC) Low RC Low conc. of phospholipid micelles containing rhTF in Tris-Hepes-NaCl buffer
Reagent C High: 1 x 0.5 TGA reagent C (RC) High RC High conc. of phospholipid micelles containing rhTF in Tris-Hepes-NaCl buffer
Reagent D: 1 x 1.5 TGA reagent D (RD) RD conc. of phospholipid micelles in TrisHepes-NaCl buffer
Control High: 1 x 1 TGA control high (CH) Human plasma with increased thrombin generation, lyophilized.
Control Low: 1 x 1 TGA control low (CL) Human plasma with decreased thrombin generation, lyophilized.
Each reagent is available separately.

Materials Required but Not Supplied

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Pipettes
Distilled water
Microtiter plates suitable for fluorescence measurement (we recommend black NUNC Maxisorp REF 475515)
Fluorimeter, fluorescence reader (96-well format), ~360 nm/~460 nm (excitation/emission) with suitable software to monitor changes of fluorescence over time

Preparation

PREPARATION OF SAMPLES
In the TGA assay citrated plasma, we recommend CTAD tubes, (platelet rich, platelet poor or platelet free) can be used, depending on the specific application.
For plasma separation, mix 9 parts of venous blood and 1 part sodium citrate solution (0.11 mol/L) and centrifuge for 15 minutes at a RCF of at least 2.500 x g.
For special requirements, preparation of other plasmas might be necessary:
- for platelet rich plasma (PRP) centrifuge for 5 minutes at 100 x g and carefully pipette off the obtained PRP;
- for platelet poor plasma (PPP) centrifuge PRP for 10 minutes at 1.500 x g and carefully pipette off the obtained PPP;
- for platelet and micro particle free plasma (PFP), centrifuge PPP for 30 minutes at 15.000 x g and carefully pipette off the obtained PFP or use the Technoclone micro particle filtration unit.

PREPARATION OF REAGENTS
The lyophilized reagents must be dissolved in the volume of distilled water indicated on the vials. All reconstituted reagents should reach room temperature before use.
After exactly 20 minutes of reconstitution time and thorough mixing (Vortex), reagents are ready to use.
For standardization tests a reconstitution time of 30 minutes is recommended for controls.

Assay Protocol

Samples and dissolved reagents should reach room temperature before use.
1.) Thrombin calibration curve

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The thrombin calibration curve has to be done separately from sample measurement.
Concentration of the thrombin calibrator (CAL) is lot dependent, consult the label on the vial. The thrombin calibrator is diluted with TGA buffer as indicated in the table below:
1st dilution (1:2): (STD 1) 200 µL Thrombin Calibrator (CAL) + 200 µL TGA buffer (BUF)
2nd dilution (1:4): (STD 2) 100 µL 1st dilution + 100 µL TGA buffer (BUF)
3rd dilution (1:20): (STD 3) 20 µL Thrombin Calibrator (CAL) + 380 µL TGA buffer (BUF)
4th dilution (1:200): (STD 4) 20 µL 3rd dilution + 180 µL TGA buffer (BUF)
All calibrator dilutions have to be measured in duplicate.
Add reagents in the following sequence:
40 µL calibrator dilution (STD 1 - STD 4)
50 µL TGA substrate (SUB)
measure for 10 min in 30 sec intervals at 37°C
Start reading of the plate/strip immediately after pipetting the substrate.
ONLY ONE CALIBRATION CURVE HAS TO BE DONE FOR EACH LOT !
2.) Sample measurement
The reagents have to be added in the following sequence:
Reagent TGA RB TGA RC Low TGA RC High TGA RD *
sample 40 µL 40 µL 40 µL 20 µL
RB 10 µL - - -
RCL - 10 µL - -
RCH - - 10 µL -
TGA RD - - - 30 µL
TGA SUB 50 µL 50 µL 50 µL 50 µL
measure for 60 min (for FVIII inhibitor therapy 90 - 120 min) in 1 minute measurement intervals at 37°C
Start reading of the plate immediately after pipetting the substrate.
A reagent substrate mixture can be prepared in advance.
Preparation of the mixture:
The mixture of reagent and substrate should be done in a 1+5 proportion for RC Low and RC high and in a 3+5 proportion for RD.
The mixture can be aliquoted and frozen at -20°C.

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When reagent/substrate mixture is used the reagents have to be added to the plate in the following sequence:
Reagent RB RC Low RC High TGA RD*
sample 40 µL 40 µL 40 µL 40 µL
reagent/substrate mixture 60 µL 60 µL 60 µL 60 µL
measure for 60 min (for FVIII inhibitor therapy 90 - 120 min) in 1 minute measurement intervals at 37°C
Start reading of the plate immediately after pipetting the reagent/substrate mixture.
Attention ! We recommend to measure duplicates for each samples.
Attention ! The lamp of the Biotek Readers is loosing intensity the longer the machine is switched on. We recommend to run only 2 consecutive runs and to switch off the reader for ~ 3 hours before you start the next run.
* ATTENTION: different pipetting scheme for Reagent D

Analysis

THROMBIN CALIBRATION CURVE
Using the provided evaluation software, RFU data (relative fluorescence units) measured by the fluorimeter for the different thrombin concentrations are converted into a thrombin calibration curve.
This thrombin calibration curve is then used by the provided software to calculate nM thrombin present in the sample at a given time.
STANDARDIZATION
The thrombin calibrator is calibrated against the thrombin Reference Preparation of the WHO.
ANALYSIS OF SAMPLES
The provided evaluation software calculates thrombin generation in the sample over time and the results are given in nM thrombin generated in the sample for each point of time during the whole coagulation process. Upon initiation of coagulation in the samples by addition of CaCl2 and the phospholipid/tissue factor mixture, generation of thrombin is initiated after a lag period; thereafter thrombin generation per minute increases, reaching a maximum of thrombin generated and decreases thereafter. The pattern seen resembles the figure provided below:
The following parameters can be used as readout in our software:
1. Lag phase from the time point when the TGA reagent including CaCl2 is added until the first burst

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in thrombin formation
2. Slope: Steepest rate of thrombin formation per minute.
Calculated by the software as velocity index
Velocity Index= peak thrombin / peak time lag time
3. Peak thrombin: Maximal concentration of thrombin formed
4. AUC: Area under the curve
Slope and peak thrombin can depend on the amount of phospholipids present in the sample. Since the provided amount of phospholipids in reagents (RC Low, RC High or RD) is limited this value is determined in PPP by the number and composition of micro particles present in the sample. In most instances there is a good correlation between slope and peak thrombin. Both parameters also depend on the amplification of initial thrombin generated and are higher in states of thrombophilia and decreased during anticoagulation therapy or in patients with bleeding disorders.

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