CY-QUANT™ MV-TF Activity

Kit to evaluate microvesicle-associated TF-dependent procoagulant activity

96 tests per kit **Ref. 7503**

BioCytex

For research use only

1 - INTRODUCTION

Tissue factor (TF) is a transmembrane glycoprotein that is considered the primary initiator of the extrinsic coagulation pathway.

Although various forms of TF have been described, procoagulant potential is clearly associated with the membrane form. Membrane-bound TF is found on most cells that do not come into contact with the blood, such as epithelial cells or nerve cells; it is also expressed on some activated blood cells (monocytes) and cancer cells, and in particular on microvesicles (MVs) derived from these cells (1).

The first step in the activation of the extrinsic coagulation pathway is formation of the TF/factor VII (FVII) complex. Upon binding to TF, FVII is rapidly activated, forming FVIIa. The TF/FVIIa complex then activates factor X (FX), generating FXa. Once FXa has been released from the TF/FVIIa/FXa complex, it binds to factor Va (FVa) on the surface of platelets or platelet MVs, leading to formation of the prothrombinase complex, which converts prothrombin into thrombin. The TF/FVIIa complex can also activate factor IX (FIX), producing FIXa, resulting in thrombin generation, thus amplifying clot formation (2).

2 - PRINCIPLE

The CY-QUANT™ MV-TF Activity assay measures FXa generation due solely to TF concentration in the sample (3):

- the sample is incubated for 30 minutes at 37°C, with or without an antibody that blocks TF activity (R2a, R2b).
- after adding a mixture of coagulation factors (FVII+FX; R3), FXa is generated during a 2-hour incubation at 37°C.
- the quantity of FXa produced is determined after stopping the reaction (R5) and adding FXa substrate (R6) (measuring para-nitroaniline (pNA) release at 405 nm).

The quantity of FXa generated is proportional to the TF concentration in the sample.

3 - REAGENTS PROVIDED

- **Reagent R1:** 1 vial containing 15 mL of 10X washing buffer
- **Reagent R2a:** 1 vial containing 1.5 mL of negative control (non-blocking MAb)
- **Reagent R2b:** 1 vial containing 1.5 mL of anti-TF control (blocking MAb)
- **Reagent R3:** 2 vials of "FVII + FX mix", lyophilised
- **Reagent R4:** 1 vial containing 3 mL of dilution buffer for R3
- **Reagent R5:** 1 vial containing 2.5 mL of stop solution
- **Reagent R6:** 1 vial containing 8 mL of chromogenic FXa substrate
- **Reagent R7:** 2 vials of calibrator, lyophilised
- **Reagent R8:** 2 vials of low-activity control, lyophilised
- **Reagent R9:** 2 vials of high-activity control, lyophilised

4 - PRECAUTIONS

- Observe good laboratory practice.
- Comply with the local regulations in force concerning waste disposal.
- Treat biosamples as potentially infectious material.
- Only use reagents from the same kit or the same lot.
- Use the same type of plate throughout to ensure comparable results.

- Reagent 5 – Stop solution
  - **H333**: May be harmful if inhaled
  - **P261**: Avoid breathing vapours
  - **P280**: Wear protective gloves/protective clothing/eye protection/face protection
- Reagent 6 – Chromogenic FXa substrate
  - **H317**: May cause an allergic skin reaction
  - **H412**: Harmful to aquatic life with long-lasting effects
  - **H402**: Harmful to aquatic life
  - **P273**: Avoid release to the environment
  - **P280**: Wear protective gloves/protective clothing/eye protection/face protection
  - **P302+P352**: IF ON SKIN: Wash with plenty of soap and water

5 - MATERIALS REQUIRED BUT NOT PROVIDED

- 96-well microplate reader with a 405 nm filter and kinetic reading capability at 37°C.
- Deionised or distilled water, preferably sterile, equilibrated to room temperature (RT).
- Timer.
- Multichannel pipettes, pipettes with disposable tips.
- Vortex mixer.
- Suitable 96-well flat-bottom microplates, such as Greiner (ref. 655101).
- 1 mL polypropylene microtubes suitable for 96-well systems, such as Starlab (ref. E1720-8000).
- Plastic film to seal the microplate.
- 37°C incubator.
- Reagent reservoir for multichannel pipette.

6 - REAGENT RECONSTITUTION AND STORAGE

When stored at 2-8°C in their original state, the reagents are stable up to the expiry date indicated on the packaging.

All reagents must be allowed to equilibrate to room temperature (RT: +18 to +24°C) for at least 30 minutes before use.

Note: Do not freeze the reagents.

- **Reagent R1:**

Stability after opening: stable for 2 months at 2-8°C in its original vial, if no contamination.

Prepare a 1:10 dilution in deionised or distilled water (R1-1X).

Prepare the volume required for the series to be tested.

Stability after dilution: 2 months at 2-8°C.

- **Reagents R2a, R2b, R4, R5 and R6:**

Ready to use.

Stability after opening: stable for 2 months at 2-8°C in their original vials, if no contamination.

- **Reagent R3:**

Reconstitute each vial with 1.5 mL of reagent R4, then mix the contents for 5 seconds on a vortex mixer to obtain a homogeneous solution.

Stability after reconstitution: stable for 4 hours at 18-24°C in its original vial.

- **Reagents R7, R8 and R9:**

Reconstitute each vial with 0.5 mL of deionised or distilled water, then mix the contents for 5 seconds on a vortex mixer to obtain a homogeneous solution.

Stability after reconstitution: stable for 4 hours at 18-24°C in its original vial.

7 - SAMPLE PROCESSING

The CY-QUANT™ MV-TF Activity assay protocol can be performed on a suspension of washed purified MVs derived from plasma, culture supernatant or any other biological or synthetic material bearing TF membrane.
Plasma MVs are assayed using PFP (platelet-free plasma) taken into a citrate tube.

8 - TEST PROCEDURE

8.1 - Sample preparation

Example: preparation of samples from PFP

- Dilute 500 µL of PFP 1:2 with 500 µL of reagent R1-1X.
- Centrifuge for 1 hour at 24000 g.
- Carefully remove the supernatant (SN), leaving behind about 50 µL above the pellet.
- Take up the pellet with 1 mL of reagent R1-1X and homogenise using a vortex mixer.
- Centrifuge for 1 hour at 24000 g.
- Carefully remove all the SN, leaving only the pellet behind.
- Resuspend the pellet with 125 µL of reagent R1-1X.

The assay is performed on MVs recovered in the pellet.

NB: samples prepared in this way have been concentrated 4 folds (500/125). This concentration factor must be taken into account when determining the activity of the sample.

8.2 - Protocol

Calibration

A series of 8 calibration dilutions is prepared from the calibrator, R7, diluted with reagent R1-1X. The first solution is undiluted calibrator, and the remaining 7 solutions are prepared through two-fold serial dilution of this calibrator.
Two **blank** points are obtained using reagent R1-1X.

Preparation of calibration dilutions

Place 8 tubes, numbered D1 to D8, in a rack.
- Add 200 µL of reagent R1-1X to each of tubes D2 to D8.
- Add 200 µL of reagent R7 (calibrator) to tubes D1 and D2.
- Homogenise tube D2 on a Vortex mixer and pipette 200 µL into tube D3.
- Perform the subsequent dilutions by repeating this last step, as indicated in the table below, up to dilution D8.

Change the tip after each pipetting operation to avoid any carry-over.

Dilution	Volume	Volume R1-1X
D1	200 µL of R7	-
D2	200 µL of R7	200 µL
D3	200 µL of D2	200 µL
D4	200 µL of D3	200 µL
D5	200 µL of D4	200 µL
D6	200 µL of D5	200 µL
D7	200 µL of D6	200 µL
D8	200 µL of D7	200 µL

Controls

The high and low positive controls are processed in the same way as samples, without need of sample preparation step described in section 8.1 above.

Samples

Samples containing too high a concentration of TF will need to be diluted as appropriate before testing.

Assay

	Calibration dilutions	Sample / Controls	Volume
Loading of calibration dilutions and samples / controls	Load the 8 dilutions (D1 to D8) + the 2 blank wells (R1-1X)	Load the sample (or control) into 2 wells	**60 µL**
Specific blocking of TF activity	Put reagent **R2a** in the 8 dilutions (D1 to D8) + the 2 blank wells	Put reagent **R2a** in the 1st well & reagent **R2b** in the 2nd well	**20 µL**
	Carefully mix the contents of each well by pipetting up and down 3 times		
Incubation	Cover the wells with plastic film and incubate for **30 minutes** at 37°C		
FXa generation	Reagent **R3**		**20 µL**
	Carefully mix the contents of each well by pipetting up and down 3 times		
	Cover the plate with plastic film and incubate for **2 hours** at 37°C		
Stop the reaction	Reagent **R5**		**20 µL**
	Carefully mix the contents of each well by pipetting up and down 3 times		
Development	Reagent **R6**		**80 µL**
Reading	Measure OD at 405 nm in kinetics mode at 37°C, for 15 minutes (1 measurement per minute)		

NB:
Prevent bubble formation in the wells throughout the assay. If bubbles are present, get rid of them before the plate is read using clean tips.

9 - RESULTS

9.1 - Calibration and sample results

Some readers come with software enabling results to be expressed directly as maximum velocity, Vmax in milli Optical Density Unit per minute (mODU/min). If this is not the case for the reader used, the OD405nm kinetics should be exported and Vmax values calculated over the 15 kinetics points using the Excel formula: "SLOPE(known_y_values; known_x_values)".

Results can be processed using a calculation template file, available on request from BioCytex or from www.biocytex.com.

Results can also be processed without the file by following the steps described below:

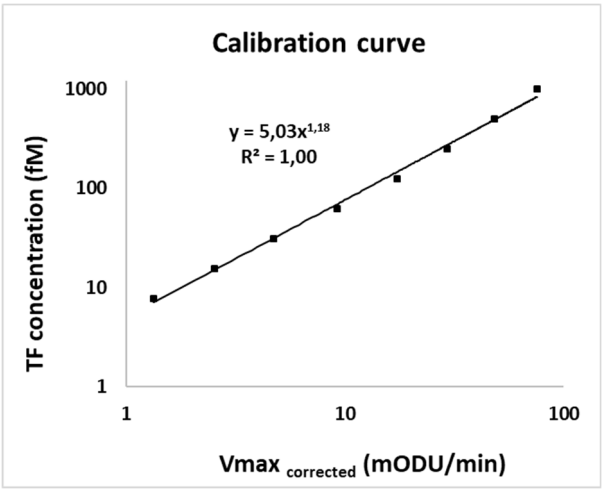
9.1.1 - Calibration:
- Obtain the results expressed directly by the software as Vmax values or determined using Excel
- These Vmax values are then corrected by subtracting the Vmax corresponding to background noise (mean of the 2 blank wells).

$$Vmax\ corrected = Vmax - Vmax\ blank$$

- Generate a log-log graph in Excel, plotting the corrected Vmax values of the 8 calibration points along the x-axis and the corresponding TF concentrations (fM) along the y-axis, derived from the concentration of calibrator R7 indicated on the calibration assay value insert.
- Plot the calibration curve, adding a "power" trendline on the dataset, where x = Vmax and y = TF concentration (fM).
- Display the equation of the curve: $y = ax^b$
Where: $1.0 \leq b \leq 1.30$ and $r^2 \geq 0.98$

NB:
If b and/or r^2 fall outside these values, the results must be interpreted with caution.

Example of a calibration curve:



9.1.2 - Samples:
- Calculate the specific Vmax value for samples/controls by subtracting the Vmax value independent of TF (obtained from wells treated with blocking anti-TF antibody) from the total Vmax value (obtained from wells treated with non-blocking control antibody):

$$Vmax\ specific = Vmax\ total - Vmax\ independent\ of\ TF$$

- Use the calibration curve to derive TF concentrations (fM) for samples/controls from the corresponding specific Vmax values.
Round the results up or down to the nearest integer.
If the sample was prepared as described in section 8.1, the measured activity will need to be adjusted for this concentration factor (see the note in section 8.1).

9.2 - Internal controls
The correlation between the concentration values obtained for the high and low positive controls and the ranges indicated on the calibration assay value insert, validates to the accuracy of the results.

10 - CARACTERISTICS OF THE METHOD
The measurement zone of the method ranges from 10 fM to 1500 fM. Taking into account the concentration factor applied for a prepared plasma sample as described in paragraph 8.1, this would correspond to an area range from 2,5 fM to 375 fM.

11 - LIMITATIONS
Related to the assay
- Avoid any bacterial contamination of the reagents or plate.
- Temperature: the temperature must be maintained and controlled at 37 ± 0.5°C during incubation steps.
- TF concentration may be overestimated for samples with a high phospholipid content. It is indicated by weaker inhibition of the signal by the blocking MAb.
- A high baseline tissue factor pathway inhibitor (TFPI) concentration in a sample can affect the assay result despite MV extraction and washing.

Related to plasma sample preparation
- The assay can be affected by pre-analytical conditions (blood sampling, transport, ageing, etc.).
- The assay can be affected by the PFP preparation process.
- The assay can be affected by the pellet preparation process.

12 - REFERENCES
1) Morrissey JH. 2003. Tissue factor: in at the start... and the finish? Journal of Thrombosis and Haemostasis; 5:878–80.
2) Mackman, N. 2004. Role of tissue factor in hemostasis, thrombosis, and vascular development. Arteriosclerosis, Thrombosis, and Vascular Biology; 24:1015-1022
3) Vallier L. et al. 2019. Increasing the sensitivity of the human microvesicle tissue factor activity assay. Thrombosis Research 182;64-74.

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