

ZYMUPHEN MP-Activity

(# 521096)

Functional assay for the measurement of microparticles' procoagulant activity in plasma

For in vitro research use only

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INTENDED USE:

The ZYMUPHEN MP-Activity kit is a functional assay proposed for the measurement of microparticles' procoagulant activity in human plasma, in vitro, using automated or manual method.

APPLICATIONS:

Assay of microparticles in some clinical contexts such as cardiovascular diseases, malignancy, infectious or inflammatory pathology.

ASSAY PRINCIPLE:

The diluted assayed plasma sample, supplemented with calcium, Factor Xa and thrombin inhibitors, is introduced into one of the microplate wells coated with Streptavidine and biotinylated Annexin V, then incubated. Following a washing step, the Factor Xa-Va mixture containing calcium, then the purified prothrombin, are introduced. When present in the tested sample, microparticles bind to Annexin V and expose their phospholipids surface, thus allowing to FXa-FVa, in presence of calcium, to activate prothrombin into thrombin. The phospholipids concentration is then the limiting factor. There is a direct relationship between the phospholipids concentration and the amount of thrombin generation, which is measured via its specific activity on the thrombin substrate. The reaction is stopped with 2% Citric Acid and Absorbance is measured at 405 nm (A405).

TESTED SAMPLES:

Citrated human plasma, prepared from citrated blood collected using conditions, which avoid any activation (refer paragraph "sample collection and preparation").

REAGENTS:

- COAT:** Micro ELISA plate, containing 12 strips of 8 wells, coated with Streptavidine and biotinylated Annexin V, then stabilized; the plate is packed in an aluminium pouch hermetically sealed in presence of a desiccant.
 - SD-MP:** 2 vials of 50 ml of **Sample Diluent (SD-MP)**, containing FIIa and FXa inhibitors, ready to use.
 - R1:** 2 vials of **Bovine FXa-FVa** mixture, containing calcium, lyophilised (to be reconstituted with 6 ml distilled water).
 - R2:** 2 vials of purified **human Prothrombin**, lyophilised (to be reconstituted with 3 ml distilled water).
 - R3:** 2 vials of **Thrombin specific chromogenic substrate** (IIa Chrom. Substrate), lyophilised (to be reconstituted with 3 ml distilled water).
 - Cal:** 2 vials of **Calibrator**, lyophilised, prepared from a washed and lysed platelet concentrate. When restored with 2 ml of **Sample Diluent (SD-MP)**, the ready to use calibrator, with a concentration "C" (in nM) of microparticles, established respectively to an internal standard, is obtained. This concentration is expressed in nM Phosphatidyl Serine (PS) equivalent.
 - CI:** 1 vial of 0.5 ml of plasma **Control I**, lyophilised.
 - CII:** 1 vial of 0.5 ml of plasma **Control II**, lyophilised
- Note:** The microparticles concentration of the calibrator and controls can vary according to the lot used, and are precisely indicated for each lot on the flyer provided with the kit.
- WS-MP:** 1 vial of 50 ml of 10 fold concentrated **Wash Solution**.
 - CA:** 1 vial of 6 ml of 2% **Citric Acid**, stop solution, ready to use.

Note: Use only components from a same kit lot number. Do not mix components from different lots when running the assay.

Cautions:

- Bovine plasma used for the preparation of R1 and BSA, was tested for the absence of infectious agents, and collected from animals free from BSE. R2, Cal, CI and CII are prepared with human plasma, tested with registered methods and found negative for HIV antibodies, HBs Ag and HCV antibodies. However, no assay may warrant the total absence of infectious agents. Any product of biological origin must then be handled with all the required cautions, as being potentially infectious.
- Buffers (SD-MP, WS-MP) contain sodium azide (0.9g/L), which may react with lead and copper plumbing to form highly explosive metal azides. Flush with large volumes of water when discarding into a sink.
- All the required cautions must be respected in order to avoid any risk of ingestion or accidental introduction of the reagents into body. In case of skin contact, wash extensively with water. In case of contact with a wound, address to the appropriate medical service, and indicate the biological origin and the nature of the product

REAGENTS AND EQUIPMENT REQUIRED BUT NOT PROVIDED:

- **1-channel pipettes** at variable volumes from 0 to 20µl, 20 to 200µl and 200 to 1000µl, and **8-channel** or **repeating pipette** allowing dispensing 50-300 µl.
- **Micro ELISA plate washing equipment.**
- **Micro ELISA plate reader** with a wavelength set up at 405 nm.
- **Stop watch.**
- **Incubator set up at 37°C**

- **Distilled water**, preferentially sterile.

REAGENTS PREPARATION, STORAGE AND STABILITY:

In their original packaging box, before use, when stored at 2-8°C, the unopened reagents are stable until the expiration date printed on the box.

- Micro ELISA plate (COAT):** open the plastic pouch and take off the required amount of 8 well strips for the test series. When out of the pouch, the strips must be used within 30 minutes. Unused strips can be stored at 2-8°C for 4 weeks in their original aluminium pouch, in presence of the desiccant, hermetically closed and protected from any moisture, and stored in the provided microplate storage bag (minigrip).
- Sample Diluent (SD-MP):** It is ready to use. When open, it can be used for 4 weeks, stored at 2-8 °C, and provided that any bacterial contamination is avoided during use. This reagent contains 0.05% Kathon CG.
- R1:** Reconstitute each vial with exactly 6 ml of distilled water; Shake thoroughly until complete dissolution of the content (vortex). Incubate at room temperature (18-25°C) for 30 min, and at least for 15 min at 37°C before use, while shaking the vial from time to time. Homogenise the content before each use. Following reconstitution, the reagent is stable for 24 hours at 2-8°C, 8 hours at room temperature (18-25°C), or 2 months at - 20 °C or below.
- R2:** Reconstitute each vial with exactly 3 ml of distilled water; Shake thoroughly until complete dissolution of the content (vortex). Incubate at room temperature (18-25°C) for 30 min, and at least for 15 min at 37°C before use, while shaking the vial from time to time. Homogenise the content before each use. Following reconstitution, the reagent is stable for 24 hours at 2-8°C, 8 hours at room temperature (18-25°C), or 2 months at - 20 °C or below.
- R3:** Reconstitute each vial with exactly 3 ml of distilled water; Shake thoroughly until complete dissolution of the content (vortex). Incubate at room temperature (18-25°C) for 30 min, and at least for 15 min at 37°C before use, while shaking the vial from time to time. Homogenise the content before each use. Following reconstitution, the reagent is stable for 1 month at 2-8°C, 72 hours at room temperature (18-25°C), or 2 months at - 20 °C or below.
- Calibrator:** restore each vial with 2 ml **Sample Diluent (SD-MP)** in order to obtain the ready to use calibrator containing "C" (nM) of microparticles. This concentration is expressed in nM Phosphatidyl Serine (PS) equivalent. Following reconstitution, the calibrator is stable for 24 hours at 2-8°C, 8 hours at room temperature (18-25°C), or 2 months at - 20 °C or below.
- CI:** restore each vial with 0.5 ml distilled water. Following reconstitution, the control is stable for 24 hours at 2-8°C, 8 hours at room temperature (18-25°C), or 2 months at - 20 °C or below.
- CII:** restore each vial with 0.5 ml distilled water. Following reconstitution, the control is stable for 24 hours at 2-8°C, 8 hours at room temperature (18-25°C), or 2 months at - 20 °C or below.
- Wash Solution (WS-MP):** If necessary, incubate the vial for 15-30 minutes in a water bath at 37°C until complete dissolution of solids when present. Shake the vial and dilute the amount required 1:10 in distilled water (the 50 ml contained in the vial allow to prepare 500 ml of Wash Solution). The Wash Solution must be stored at 2-8°C in its original vial and used within 8 weeks following opening. The diluted Wash Solution must be used within 7 days, when protected from any contamination and stored at 2-8°C. This reagent contains 0.05%Kathon CG.
- Citric Acid 2% :** It is ready to use.

Note: Bring the kit at room temperature, at least 30 min. before use. Store the unused reagents at 2-8°C.

Stability studies performed at 30°C show that the reagents keep their performances and can be shipped at room temperature without any damage.

Cautions:

- R1, R2, R3 and Cal and controls vials are closed under vacuum. Remove carefully the stopper, in order to avoid any loss of powder when opening the vials.
- To incubate the reconstituted vials, for 30 min. at RT, allows stabilising the reagents, and obtaining a homogeneous reactivity over time.
- In order to improve stability, reagents must be closed with their original screw cap following each use (white cap for R1, blue cap for R2, yellow caps for R3 substrate, red cap for the calibrator (Cal), green cap for the controls, white caps for the buffers).
- Reagents must be handled with care, in order to avoid any contamination during use.
- If the substrate becomes yellow, this indicates the presence of a contaminant. It must be rejected, and a new vial must be used.
- **Buffers (SD-MP, WS-MP) contain sodium azide (0.9g/L), which may react with lead and copper plumbing to form highly explosive metal azides. Flush with large volumes of water when discarding into a sink.**
- Citric Acid, although diluted to 2%, is caustic. As for any similar chemical, handle Citric Acid with great care. Avoid any skin and eye contact. Wear protection glasses and gloves when handling.

ASSAY PROTOCOL:

Sample collection and preparation:

Blood plasma (9 vol.) must be collected through a frank venipuncture, on 0.109M (or 0.129M) citrate anticoagulant (1 vol.); plasma supernatant must be rapidly decanted (**within 2 hours**) following a **15 min. centrifugation at 1,500 g at room temperature (note: samples must never be stored or centrifuged at 4°C)**; plasma supernatant is then again rapidly centrifuged for **2 min. at 13,000 g at room temperature**. Plasma is obtained by collecting the supernatant, avoiding any contact with the platelet pellet. Plasma should be tested within **4 hours**, or stored frozen at -80°C or below for up to 6 months, and thawed for 15 min. at 37°C just before use. Thawed specimen must be tested within 4 hours, when stored at room temperature. Thawed specimen must never be refrozen.

Tested plasma or control:

Samples or controls are tested at **1:20** dilution in Sample Diluent (**SD-MP**). When high amounts of microparticles are expected, ($> 10^6$ nM), samples must be assayed more diluted (i.e. dilution "D")

Calibration curve:

Using the Calibrator, at "C" nM, provided in the kit, prepare the following standard solutions.

Microparticles concentration (nM)	C	C/2	C/4	C/10	C/20	0
Calibrator Vol.	1 ml	0.5 ml	0.25 ml	0.1 ml	0.05 ml	0 ml
Sample Diluent Vol.	0 ml	0.5 ml	0.75 ml	0.9 ml	0.95 ml	1 ml

Mix gently until complete homogenisation.

The standard dilutions are stable for at least **4 hours** at room temperature.

Assay procedure:

Remove the required number of strips from the aluminium pouch, for the series of measures to be performed. Then put the strips in the frame provided. At least 15 min. before each respective use, incubate reagents R1, R2 and R3 at 37°C . In the different wells of the micro ELISA plate introduce the reagents and perform the various assay steps as indicated on the following table:

Reagent	Volume	Procedure
Calibrator dilutions or 1:20 diluted controls or 1:20 diluted sample or sample diluent (blank)	100 μl	Introduce the : - calibrator dilutions or - diluted controls or - diluted sample or - sample diluent (blank) into the micro ELISA plate wells (a)
Incubate for 1 hour at 37°C (b)		
Wash Solution (10 fold diluted in distilled water)	300 μl	Proceed to 5 successive washings at room temperature ($18-25^{\circ}\text{C}$) (c).
Following washing, introduce:		
R1 (reconst. with 6ml of distilled water, and stabilized at 37°C)	100 μl	Introduce R1 into the micro ELISA plate wells (c).
R2 (reconst. with 3ml of distilled water, and stabilized at 37°C)	50 μl	Introduce R2 into the micro ELISA plate wells (c).
Incubate for 10 minutes at 37°C (b)		
R3 Substrate stabilized at 37°C	50 μl	Immediately introduce the substrate into the wells (c, d). Note: The substrate distribution, row by row, must be accurate and at exact time intervals.
Let the colour to develop for exactly 3 min. at 37°C (b)(e)		
Citric Acid 2%	50 μl	Following exactly the same time intervals than for the addition of substrate, stop the colour development by introducing 2% Citric Acid (d)
Wait for 10 minutes in order to allow the colour to stabilize and measure absorbance at 405 nm (A405) , subtracting the blank value. (e).		

Note:

- Distribute calibrators, controls and tested specimen as rapidly as possible (**within 10 minutes**), in order to obtain a homogeneous kinetics for the whole plate.
- Avoid letting the plate in the bright sunlight and more particularly during colour development. Incubate the plates in an incubator at 37°C .
- Never let the plates empty between the addition of reagents or following the washing step. The next reagent must be added within 3 minutes, in order to prevent the plate from drying, which could damage the immobilized components. If necessary, keep the plate filled with Wash Solution and empty it just before the introduction of the next reagent. The washing instrument must be adjusted in order to wash the plates gently, and to avoid a too drastic emptying, which could lower plate reactivity. R1, R2 and R3 must be incubated at 37°C before use.
- For addition of the substrate, the time interval between each row must be accurate and exactly determined. It must be the same when stopping the reaction with Citric Acid.
- The assay can be used with a kinetics mode and ΔA_{405} measured between 20 seconds and 2 min., for example, on a microplate reader allowing plate incubation at 37°C .

LIMITATIONS OF THE ASSAY:

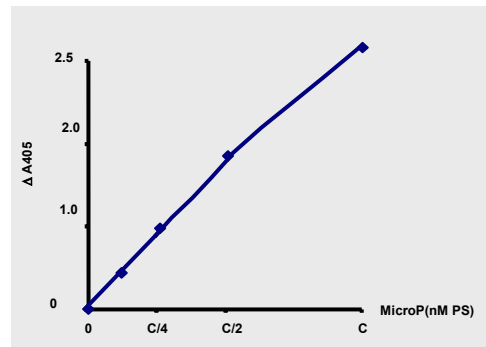
- Blood activation, during plasma collection and preparation, and presence of residual platelets in plasma, can induce microparticles' release and elevated concentrations measurement in plasma.
- In order to get the optimal performances of the assay, the procedural instructions must be strictly adhered to.

EXPRESSION OF RESULTS:

- Results are expressed according to the A405 values obtained for samples, and microparticle concentrations are calculated using the calibration curve.
- Using a linear graph paper, the calibration curve is obtained by plotting the microparticle concentration in nM on the abscissae and the corresponding A405 on the ordinates.
- Only the calibration curve generated for the series of measures performed must be used.
- The microparticle concentration, obtained for the sample tested is deduced from the curve, by multiplying the measured concentration by the dilution factor (i.e. 20 or "D").
- Alternatively, ELISA software (i.e. Dynex, Biolise, etc...), can be used for the calculation of microparticle concentrations.
- In order to get the optimal performances of the assay, the procedural instructions must be strictly respected.

EXAMPLE OF CALIBRATION CURVE:

This curve is provided as an example only (for "c" corresponding to about 2.5 nM).



Only the calibration curve generated for the series of measures performed must be used.

QUALITY CONTROL:

Concentrations obtained for controls must be within the acceptance ranges indicated on the flyer provided in the kit. If controls are out of these ranges check carefully the assay conditions and re-run the assay, if required.

ASSAY PERFORMANCES AND CHARACTERISTICS:

- Detection threshold: ≤ 0.05 nM.
- Intra-assay CV: 3-8%.
- Inter-assay CV: 5-10%.

EXPECTED VALUES:

Microparticles' concentration in normal human plasma is usually $\leq 5\text{nM}$ (expressed as phosphatidylserine (PS) equivalent).

PATHOLOGICAL VARIATIONS:

Plasma concentration is considered as pathological when the microparticles' concentration is > 2 fold that of normal plasmas, i.e. $\geq 10\text{nM}$. High levels of microparticles are observed in various pathological contexts such as cardiovascular diseases, malignancy, infectious or inflammatory pathology, etc...

BIOCHEMISTRY:

Microparticles from various origins are present in plasma, especially in some clinical contexts. They are generated by the activation of blood cells (platelets, endothelium, leucocytes, monocytes...). They are the consequence of disease, but can also be the cause of clinical complications, by stimulating the blood procoagulant potential. According to their origin, these microparticles can expose a procoagulant phospholipids surface and thrombogenic proteins such as Tissue Factor.

APPLICATIONS:

Assay of microparticles as a marker of cardiovascular disease, malignancy, and/or infectious or inflammatory pathology (exploration or follow-up of pathology evolution, diagnosis, prognosis of the disease recurrence, or monitoring of the therapy efficacy). **Research Use Only (RUO)**.

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