

# ZYMUPHEN™ MP ACTIVITY

Ref 521096 (96 tests)



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

**FOR RESEARCH USE ONLY.**

**NOT FOR USE IN DIAGNOSTIC PROCEDURES.**

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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.

**This kit is for research use only and should not be used for patient diagnosis or treatment.**

## SUMMARY AND EXPLANATION:

Microparticles from various origins are present in plasma, particularly in pathological situations. They are generated by the activation of blood cells (platelets, endothelium, leucocytes, monocytes...). According to their origin, these microparticles can expose a procoagulant phospholipids surface and thrombogenic proteins such as Tissue Factor.

Plasma microparticles' concentration is usually  $\leq 5$  nM in phosphatidylserine (PS) equivalent.

## ASSAY PRINCIPLE:

The assay of the microparticles, with the ZYMUPHEN™ MP-ACTIVITY kit, is performed using a microtitre plate sensitized with streptavidin and biotinylated annexin V, and then stabilized. The diluted assayed plasma sample, supplemented with calcium, Factor Xa (Fxa) and thrombin (FIIa) inhibitors, is introduced into one of the microplate wells. Following incubation and a washing step, the Factor Xa-Va mixture containing calcium are introduced, then the purified prothrombin. 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 generated, 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.

## 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**, lyophilised (to be reconstituted with 3 mL distilled water).
- **Cal:** 2 vials of **Calibrator**, lyophilised, prepared from a washed and lysed platelet concentrate. Each vial must be restored with 2 mL of **Sample Diluent (SD-MP)** to obtain a calibrator with a concentration "C" (in nM) of microparticles. This activity is expressed in nM Phosphatidyl Serine (PS) equivalent.
- **CI:** 1 vial of 0.5 mL of plasma **Control High (Control I)**, lyophilised.
- **CII:** 1 vial of 0.5 mL of plasma **Control Low (Control II)**, lyophilised
- **WS-MP:** 1 vial of 50 mL of 10 fold concentrated **Wash Solution**.
- **CA:** 1 vial of 6 mL of 2% **Citric Acid**, ready to use (stop solution).

The concentration for each controls and the calibrator is indicated on the flyer provided in the kit. The microparticles concentrations for the controls and the calibrator may vary from lot to lot. For the assay, refer to the concentration indicated on the flyer provided in the kit used. Reagent 2 and 9 contain low concentration of Sodium azide (0.9 g/L), see CAUTIONS AND WARNINGS

## CAUTIONS AND WARNINGS:

- Any product of biological origin must then be handled with all the required cautions, as being potentially infectious.
- Sodium azide may react with lead and copper plumbing to form highly explosive metal azides.
- If the substrate becomes yellow, this indicates the presence of a contaminant. It must be rejected, and a new vial must be used.
- The disposal of waste materials must be carried out according to current local regulations
- Use only reagents from kits with the same lot number. Do not mix reagents from kits with different lots when running the assay; they are optimized for each lot of kits.
- Reagents must be handled with care, in order to avoid any contamination during use. Take care to limit as much as possible any evaporation of the reagents during use, by limiting the liquid-air surface exchange.
- In order to improve stability, reagents must be closed with their original screw cap following each use.
- Stability studies for 3 weeks at 30°C show that the reagents can be shipped at room temperature for a short period without damage.
- For in vitro use

## PREPARATION AND STABILITY OF REAGENTS:

Vials are closed under vacuum. Remove carefully the stopper, in order to avoid any loss of powder when opening the vials.

- **COAT:** open the aluminium pouch and take off the required amounts 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 pouch, in presence of the desiccant, hermetically closed and protected from any moisture, and stored in the provided microplate storage plastic bag (minigrip).
- **SD-MP:**  
Ready to use. This reagent contains 0.05% Kathon CG. Stability of reagent, provided that any contamination or evaporation is avoided, kept in its original vial is:
  - 4 weeks at 2-8°C.
- **R1:** Reconstitute each vial with exactly 6 mL of distilled water, shake thoroughly for complete dissolution. Let the reagent stabilize for 30 min at room temperature (18-25°C); while shaking the vial from time to time.  
Homogenize before each use.  
Stability of reconstituted reagent, provided that any contamination or evaporation is avoided, kept in its original vial is:
  - 24 hours at 2-8°C.
  - 8 hours at room temperature (18-25 °C).
  - 2 months frozen at -20°C or below\*
- **R2:** Reconstitute each vial with exactly 3 mL of distilled water, shake thoroughly for complete dissolution. Let the reagent stabilize for 30 min at room temperature (18-25°C); while shaking the vial from time to time.  
Homogenize before each use.  
Stability of reconstituted reagent, provided that any contamination or evaporation is avoided, kept in its original vial is:
  - 24 hours at 2-8°C.
  - 8 hours at room temperature (18-25 °C).
  - 2 months frozen at -20°C or below\*
- **R3:** Reconstitute each vial with exactly 3 mL of distilled water, shake thoroughly for complete dissolution. Let the reagent stabilize for 30 min at room temperature (18-25°C); while shaking the vial from time to time.  
Homogenize before each use.  
Stability of reconstituted reagent, provided that any contamination or evaporation is avoided, kept in its original vial is:
  - 1 month at 2-8°C.
  - 72 hours at room temperature (18-25 °C).
  - 2 months frozen at -20°C or below\*
- **Cal:** Reconstitute each vial with exactly 2 mL of Sample Diluent (SD-MP) in order to obtain a ready to use calibrator containing a concentration "C" (in nM) of microparticles. This concentration is expressed in nM Phosphatidyl Serine (PS) equivalent.  
Homogenize before each use.  
Stability of reconstituted reagent, provided that any contamination or evaporation is avoided, kept in its original vial is:
  - 24 hours at 2-8°C.
  - 8 hours at room temperature (18-25 °C).
  - 2 months frozen at -20°C or below\*
- **CI:** Reconstitute each vial with exactly 0.5 mL of distilled water. Shake thoroughly for complete dissolution.
- **CII:** Reconstitute each vial with exactly 0.5 mL of distilled. Shake thoroughly for complete dissolution.  
Stability of reconstituted Controls, provided that any contamination or evaporation is avoided, kept in its original vial is:
  - 24 hours at 2-8°C.
  - 8 hours at room temperature (18-25 °C).
  - 2 months frozen at -20°C or below\*
- **WS-MP:** if necessary, incubate the vial for 15-30 minutes in a water bath at 37°C until complete dissolution of crystals, when present. Shake the vial and dilute the amount required 1:10 in distilled water (the 50 mL contained in the vial allow preparing 500 mL of Wash Solution). Following opening, the Wash Solution is stable 8 weeks at 2-8°C, when protected from any contamination. 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.
- **CA:** 2% citric acid solution, ready to use.

\*Thaw once as rapidly as possible at 37°C, adapt duration to the volume of reagent. The stability of the thawed reagent should be verified in the working conditions of the user laboratory.

## STORAGE CONDITIONS:

Unopened reagents must be stored at 2-8°C, in their original packaging box. They are then usable until the expiration date printed on the label.

## REAGENTS AND MATERIAL REQUIRED BUT NOT PROVIDED:

### Reagents:

- Distilled water.

### Materials:

- Micro ELISA plate washing equipment (and agitator).
- Spectrophotometer or automatic instrument for chromogenic assays with a wavelength set up at 405 nm (reading range up to 4u of OD).
- Stopwatch; Calibrated pipettes.

## SPECIMEN COLLECTION:

Preparation and storage of specimens must be performed according to the current local regulations.

### Specimens:

Human plasma obtained from anticoagulated blood (trisodium citrate).

### Collection:

Blood (9 vol.) must be collected on trisodium citrate (1 vol.) (0.109M) with caution, through a net venipuncture. The first tube must be discarded.

### Centrifugation:

 (Samples must never be stored or centrifuged at 4°C)

Within 2 hours, use a validated method in the laboratory to obtain a plasma with no platelet, e.g., a minimum of 15 minutes at 2500 g at room temperature (18-25°C), then recovered plasma supernatant is rapidly centrifuged for 2 min at 13000 g at room temperature (18-25°C). Plasma is obtained by collecting the supernatant, avoiding any contact with the platelet pellet.

### Storage of plasma:

- 4 hours at room temperature (18-25°C)
- 1 month at -20°C.
- 6 months at -70°C.

Frozen plasma specimens should be rapidly thawed at 37°C, then gently mixed and tested immediately. Resuspend any precipitation by thorough mixing immediately after thawing and before testing.

## TEST PROCEDURE:

### Assay method:

1. Calibrator should be diluted using SD-MP as described in the table below in order to establish the calibration range ("C" = defined microparticles concentration):

Calibrator	C	C:2	C:4	C:10	C:20	0
Volume calibrator	1 mL	0.5 mL	0.25 mL	0.1 mL	0.05 mL	0 mL
Volume of SD-MP	0 mL	0.5 mL	0.75 mL	0.9 mL	0.95 mL	1 mL

2. The samples should be diluted using SD-MP as described in the table below:

Sample	Dilution
Control	1:20
Specimen	1:20

Run the calibration curve and test it with quality controls. Diluted sample should be tested within 1 hour when stored at room temperature (18-25°C).

Please note that the exact concentration of the calibrators and controls is indicated for each lot on the flyer provided with the kit.

3. Remove the required number of strips from the aluminium pouch, and then put the strips in the frame provided. Before each respective use, incubate reagents R1, R2 and R3 at 37 °C at least 15 min. In the different wells of the micro plate introduce the reagents and perform the various assay steps as indicated on the following table:

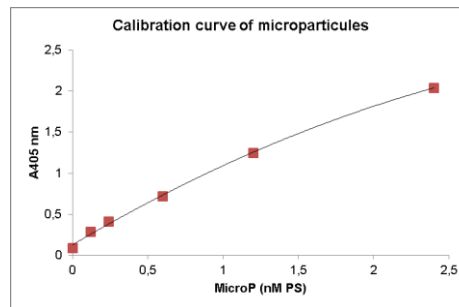
Reagent	Volume	Procedure
Calibrator or 1:20 diluted controls or 1:20 diluted sample or SD-MP (blank)	100 µL	Introduce the calibrator or 1:20 diluted controls or 1:20 diluted sample or SD-MP (blank) into the micro plate wells (a)
<b>Incubate for 1 hour at 37°C (b)</b>		
Wash Solution (10 fold diluted in distilled water before use)	300 µL	Proceed to 5 successive washings at room temperature (18-25°C) (c).
<b>Following washing, introduce:</b>		
R1 (reconst. with 6mL of distilled water, and stabilized at 37°C)	100 µL	Introduce R1 into the micro plate wells (c).
R2 (reconst. with 3mL of distilled water, and stabilized at 37°C)	50 µL	Introduce R2 into the micro plate wells (c).
<b>Incubate for 10 minutes at 37°C (b)</b>		
R3 Substrate (stabilized at 37°C)	50 µL	Immediately introduce the substrate into the wells (c, d). <b>Note:</b> The substrate distribution, row by row, must be accurate with 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 row by row, 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 (e). Subtract the blank value (f).		

### 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 for the incubation step.
- Never let the plates empty between the addition of reagents or following the washing step to preserve the immobilized proteins. The next reagent must be added within 3 minutes, in order to prevent the plate from drying. 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 on a microplate reader allowing plate incubation at 37°C. For example, measure  $\Delta A_{405}$  between 20 seconds and 2 min.
- For bichromatic readings, a reference wavelength at 620nm or 690nm can be used.

## CALIBRATION:

The calibration curve obtained below is indicated as an example only. The calibration curve generated for the series of measures performed must be used.



## QUALITY CONTROL:

Using quality controls, allows validating the calibration curve, as well as the homogeneous reactivity from run to run, when using a same lot of reagents.

Quality control must be included in each series, as per good laboratory practice, in order to validate generated results. A new calibration curve must be carried out for each test series, and when quality controls values are measured outside the acceptance range determined for the method.

Each laboratory should establish and verify its own target values, acceptance ranges and expected performances, according to the instruments and protocols used.

## RESULTS:

- 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.
- Results are expressed according to the A405 values obtained for samples, and microparticle concentrations are calculated using the calibration curve. 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, software (i.e., DYNEX, BIOLISE, etc.), can be used for the calculation of microparticle concentrations.
- Results are expressed in nM

**The results obtained should be for research purposes only and not used for patient diagnosis or treatment.**

## LIMITATIONS:

- In order to get the optimal performances of the assay, the procedural instructions must be strictly respected.
- Any reagent presenting an unusual aspect or a contamination sign must be rejected.
- Any plasma containing a coagulum or contamination must be rejected.
- Blood activation, during collection and plasma preparation, or presence of residual platelets in plasma, can induce microparticles' release and so falsely elevate the test results.

## PERFORMANCES:

- The lower limit of detection is  $\leq 0.05$  nM.
- Intra-assay CV: 3 – 8%.
- Inter-assay CV: 5 – 10%.

## REFERENCES:

- Woodhams B, et al. Stability of coagulation proteins in frozen plasma. Blood coagulation and Fibrinolysis. 2001.
- Morel O, et al. Cellular microparticles: a disseminated storage pool of bioactive vascular effectors. Curr Opin Hematol. 2004.
- Freysinet JM. Cellular microparticles: what are they bad or good for?. J Thromb Haemost. 2003.

## SYMBOLS:

Used symbols and signs listed in the ISO standard 15223-1.