

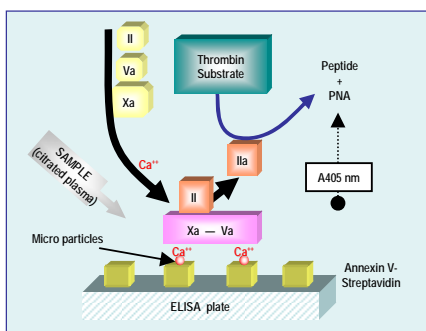
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Introduction

- In many clinical contexts associated with blood activation, microparticles are present in plasma (cardiovascular diseases, malignancy, infectious or inflammatory pathology).
- They are released from blood cells (platelets, endothelium, leucocytes, monocytes...). They are the consequence of the disease but can also be the cause of further clinical complications, by stimulating the blood procoagulant potential.
- Their surface exposes procoagulant phospholipids (i.e. Phosphatidyl Serine (PS)) and proteins (ex.: Tissue factor).
- Many applications are reported for these assays for clinical applications such as: diagnosis, prognosis or monitoring efficacy of some therapies for circulatory diseases.
- We developed an optimized assay for quantitatively measuring the procoagulant activity of microparticles in plasma, expressed as Phosphatidyl Serine (PS) equivalent (nM).
- This assay was developed from the method described by Jean Marie Freyssinet and Bénédicte Hugel (Aupeix K et al. J. Clin. Invest 1997; 99: 1546-54).

Assay Principle

- A microELISA plate is coated with streptavidin and biotinylated Annexin V (recombinant, supplied by Dr C. Reutelingperger from Maastricht, NL) then washed and stabilized.
- Diluted tested plasma or calibrator is introduced in a microwell, in presence of calcium and protease inhibitors. When present, microparticles (MPs) exposing anionic phospholipids (mainly phosphatidyl-serine, PS) bind to Annexin V.
- Following a washing step, first FXa and Va are introduced, with calcium, then prothrombin. A prothrombinase activity, dose dependent on the amount of immobilized MPs, is generated and activates Prothrombin to Thrombin.
- Generated Thrombin is measured using a specific chromogenic substrate, and absorbance is measured at 405 nm using a kinetics method or an end-point method.



Results

MPs in normal individuals (N=17)

N	Mean (nM)	Range (nM)
17	5.4	1.8-9.00

- Normal Plasmas: < 10 nMol.
- Plasmas from various pathologies: Concentration for 2 to 10 times those of the normal plasmas.

References

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- Gris JC et al. The relationship between plasma microparticles, protein S and anticardiolipin antibodies in patients with human immunodeficiency virus infection. *Thromb Haemost*, 1996, 76(1):38-45.

Conclusions

- New method for measuring microparticles procoagulant activity present in blood circulation optimized and standardized.
- Discriminant and sensitive technique with clear cut-off between normal individuals and patients with various pathologies (generating microparticles).
- Potentially very useful for the diagnosis or the prognosis of circulatory diseases' recurrence or monitoring therapy efficacy.

Microparticles' characteristics

- Long half life (\approx 6 days)
- Bind to Annexin V, in presence of Ca⁺⁺
- Released from various blood cells.
- Expose CDs :TF, TM, GP IIb-IIIa, ...

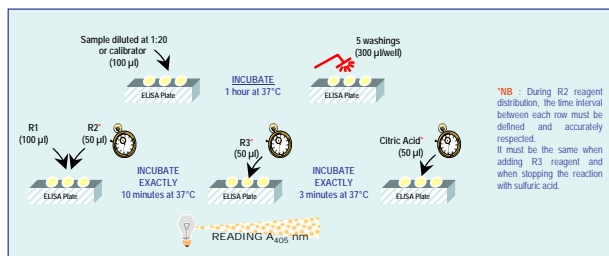
Methods

1. Plasma Preparation

Blood Collection and plasma preparation: Citrated blood, collected by a frank venipuncture, is centrifuged for 15 min. at > 2,000 g at RT, and plasma supernatant centrifuged again for 2 min. at 13,000 g at RT.

2. Assay protocol

- 100 μ l of calibrator or tested sample (citrated plasma diluted 1:20, containing Calcium, and supplemented with Factor Xa and Thrombin inhibitors), are introduced into the microplate wells coated with Streptavidin and biotinylated Annexin V, then incubated for 1 hour at 37°C.
- After washing, 100 μ l of the Factor Xa-Va-Calcium (R1) mixture are introduced into the microplate wells, then 50 μ l of purified Prothrombin (R2). Microparticles present in the tested sample are captured by Annexin V, and expose their phospholipidic surface allowing Factor Xa and Va, in presence of Calcium, to activate Prothrombin into Thrombin. The generated amount of Thrombin is then measured with the Biophen CS-01(38) substrate, (R3), and colour is read at A405 nm.
- The absorbance is measured at A405 nm.
- Calibration is performed with a washed and lysed platelet concentrate, for which the amount of microparticles is established respectively to an internal standard.



*NB : During R2 reagent distribution, the time interval between each row must be defined and accurately respected. It must be the same when adding R3 reagent and when stopping the reaction with sulfuric acid.

