



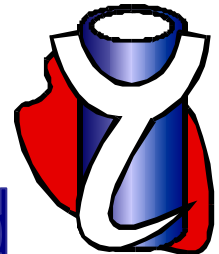
HYPHEN BioMed

**FUNCTIONAL OR IMMUNOLOGICAL ASSAYS
FOR THE MEASUREMENT OF MICROPARTICLES
ON PLASMA
(ZYMUPHEN MP-Activity & MP-TF)**

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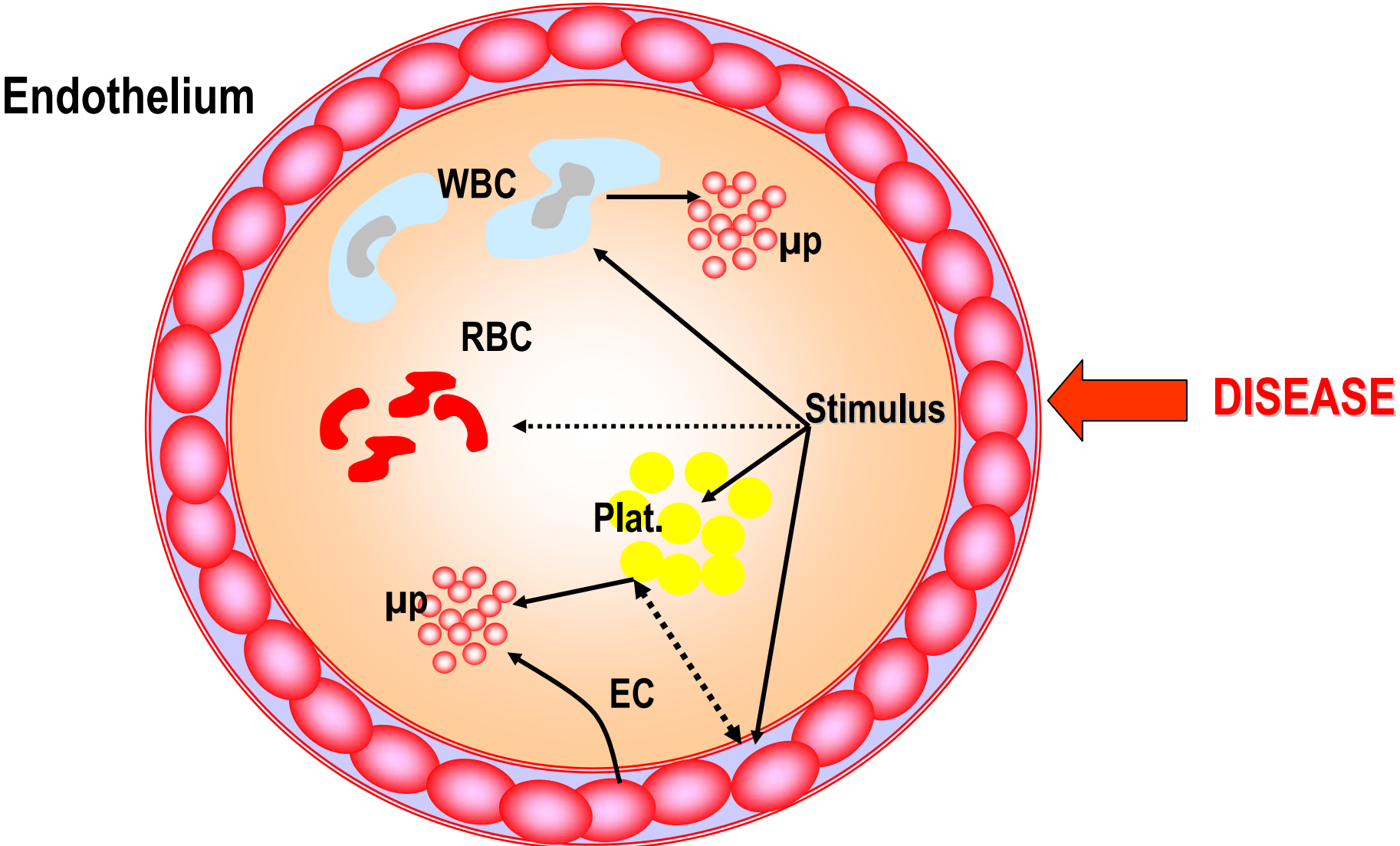
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AIM

- ❑ In some clinical contexts, microparticles are present in plasma (cardiovascular diseases, malignancy, infectious or inflammatory pathology).
- ❑ 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.
- ❑ Their surface exposes phospholipids 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.
- ❑ The assays were developed from the methods described by Jean Marie Freyssinet and Bénédicte Hugel (Aupeix K et al. J. Clin. Invest., 1997, 99, 1546-54).

GENERATION OF MICROPARTICLES



CELL ORIGIN OF MICROPARTICLES

- ❑ **Platelets**
coagulation activation
- ❑ **Endothelial cells**
auto-immune disease, TTP, coagulation activation
- ❑ **Monocytes**
inflammation, infection, ...
- ❑ **Leucocytes**
inflammation, ...
- ❑ **Lymphocytes**
diabetes, ...

MPs' 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, ...

ASSAYS

1. Functional Method :

- ❑ 100 µl of calibrator or tested sample (citrate plasma diluted 1:20, containing Calcium, and supplemented with Factor Xa and Thrombin inhibitors), are introduced into the microplate wells coated with Streptavidine and biotinylated Annexin V, then incubated for 1 hour at 37°C.
- ❑ After washing, 100 µl of the Factor Xa-Va-Calcium (R1) mixture are introduced into the microplate wells, then 50 µl 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.

1. Immunological Method:

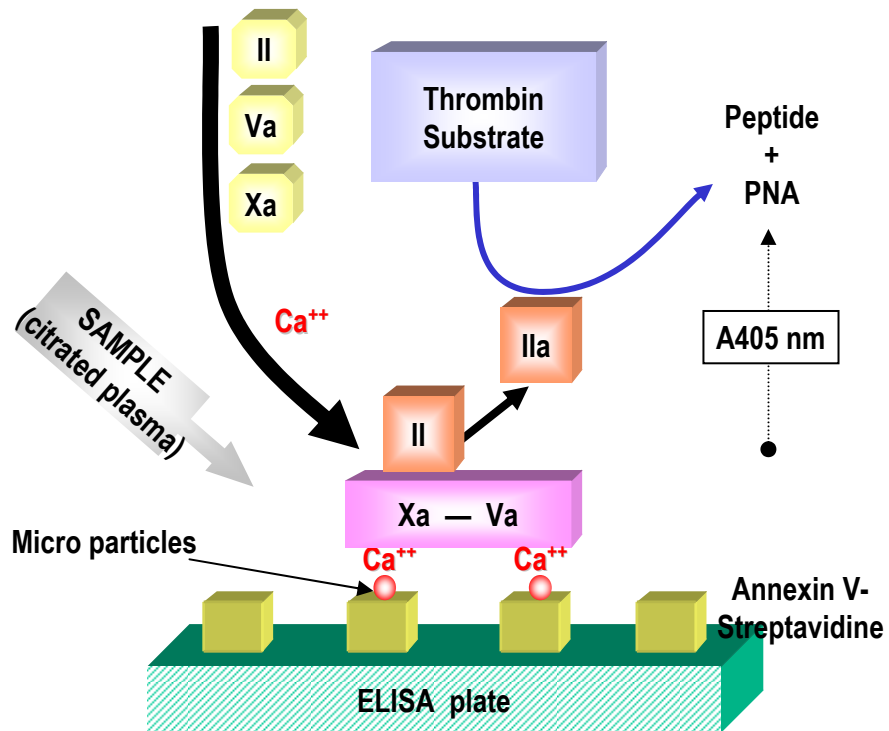
same principle for microparticles' capture.

- ❑ After washing, microparticles exposing Tissue Factor are revealed using an anti-Tissue Factor antibody coupled with peroxidase and the microplate is incubated for 1 hour at 37°C.
- ❑ After a new washing step, the peroxidase activity (which is a direct relationship of the microparticles exposing the Tissue Factor concentration) is revealed using the TMB/H₂O₂ substrate and the absorbance is measured at A450 nm.
- ❑ Calibration can be performed with recombinant relipidated Tissue Factor, or liposomes carrying the recombinant Tissue Factor.

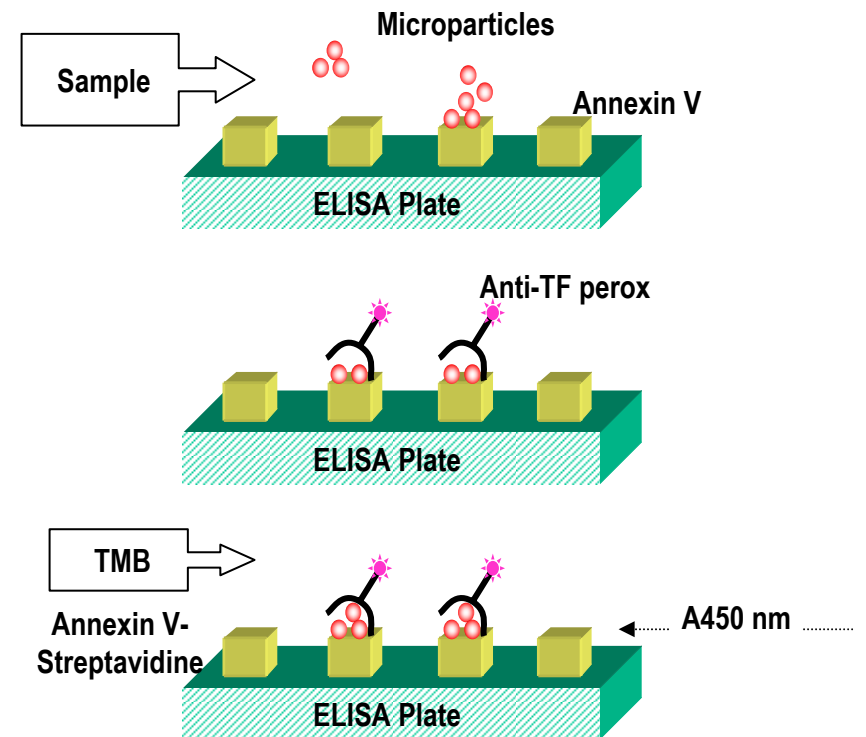
Note: For both method, assays are performed on citrated plasma, prepared from citrated blood collected, avoiding any activation.

ASSAYS' PRINCIPLE

Functional Method

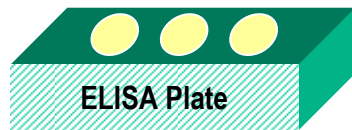


Immunological Method



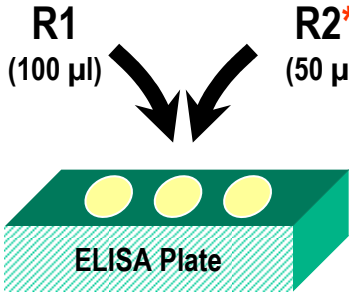
FUNCTIONAL METHOD

Sample diluted at 1:20
or calibrator
(100 μ l)

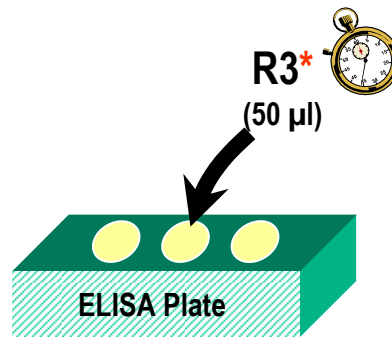


INCUBATE
1 hour at 37°C

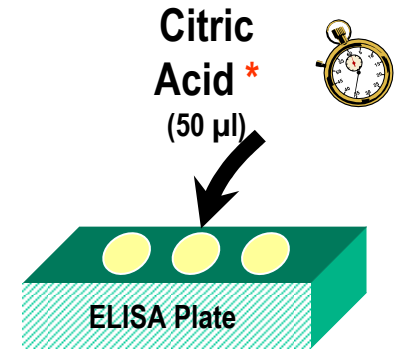
5 washings
(300 μ l/well)



**INCUBATE
EXACTLY**
10 minutes at 37°C



**INCUBATE
EXACTLY**
3 minutes at 37°C



READING A_{405} nm

***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.

PLASMA PREPARATION AND MPs' CONCENTRATION

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.

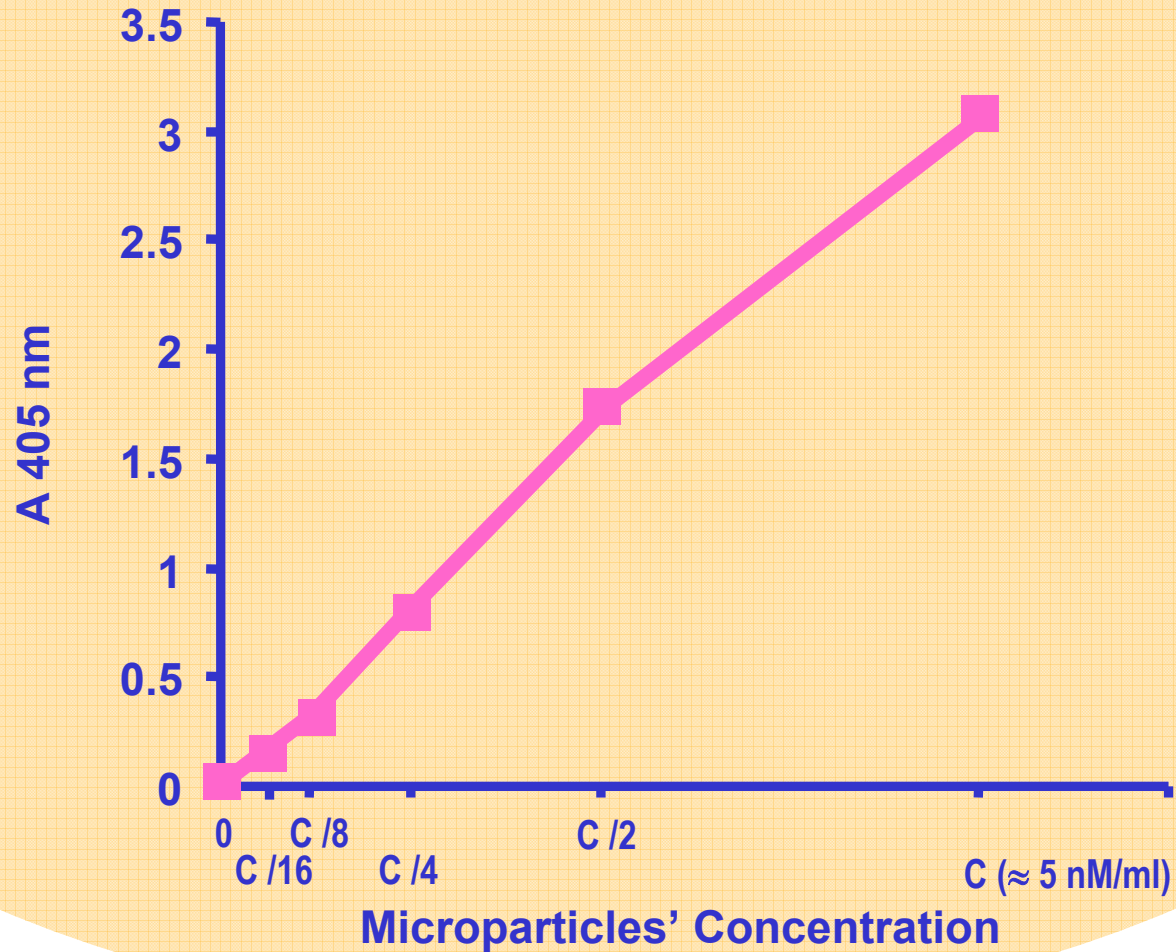
Normal Plasmas:

< 10 nMol/ml (confirmation in process).

Plasmas from various pathologies:

Concentration for 2 to 10 times those of the normal plasmas.

CALIBRATION CURVE



CONCLUSIONS

- ❑ **New methods** for exploring microparticles present in blood circulation, either by measuring their coagulant potential or by the exposed CDs (cell origin).
- ❑ **Discriminant and sensitive technique** with clear cut-off between normal individuals and patients with various pathologies (generating microparticles).
- ❑ Potentially very useful for the **diagnosis, prognosis** of the disease recurrence or **monitoring therapy efficacy**.