PLATELET GpIIb/IIIa OCCUPANCY

Evaluation of anti GpIIb/IIIa platelet anti-aggregants by flow cytometry

Kit for 10 tests

(Patent US 6 168 925 / EP 0920628) **Ref. 7001**

For Research Use Only.
Not For Use in Diagnostic Procedures.

1 METHOD

Single color flow cytometric analysis of the GpIIb/IIIa glycoprotein receptor. The total number of platelet GpIIb/IIIa receptors and the number of free GpIIb/IIIa receptors (free of anti-aggregant molecules) are determined by converting the fluorescence intensity into corresponding number of sites per platelet based on a calibrated bead standard curve.

2 REAGENTS

- **Reagent 1:** 1 x 15 mL vial, diluent, 10-fold concentrated.
- **Reagent 2a:** 1 x 200 µL vial, negative isotypic control (mouse monoclonal antibody, IgG).
- **Reagent 2b:** 1 x 200 µL vial, MAb1 reagent, anti-GpIIIa (CD 61) monoclonal antibody.
- **Reagent 2c:** 1 x 200 µL vial, MAb2 reagent, anti-GpIIIa (CD 61) monoclonal antibody.
- **Reagent 3:** 1 x 400 µL vial, calibrated bead suspension. The beads are coated with increasing and accurately known quantities of mouse immunoglobulins IgG. The number of determinants coated on each bead population is indicated in the Assay Value Insert provided in the kit.
- **Reagent 4:** 1 x 800 µL vial, staining reagent, polyclonal antibody anti mouse IgG-FITC.

WARNING
All reagents contain sodium azide as a preservative.
Reagents containing sodium azide should be discarded with care to prevent the formation of explosive metallic azides. When dumping waste materials into sinks, use copious quantities of water to flush plumbing thoroughly

3 REAGENT PREPARATION AND STORAGE

Intact kits and contents are stable until the expiration date indicated on the box label, when stored at 2-8 °C.*

- **Reagent 1****
  Stability after opening: 2 months at 2-8°C, when free of contamination.
  Prepare a 1:10 dilution with distilled water. Prepare the appropriate volume required for the samples to be tested.
  Stability after dilution: 15 days at 2-8 °C.
- **Reagents 2a, 2b and 2c**
  Ready for use.
  Stability after opening: 2 months at 2-8 °C, when free of contamination.
- **Reagent 3**
  Ready for use.
  Shake vial well, 5 seconds, to resuspend beads before opening vial.
  Stability after opening: 2 months at 2-8 °C, when free of contamination.
- **Reagent 4**
  Ready for use.
  Stability after opening: 2 months at 2-8 °C, when free of contamination.

Notes: ***** Do not freeze the kit.
****** The presence of crystals does not affect the quality of the reagent. Incubate at 37 °C until the crystals are completely dissolved.

4 SPECIMEN COLLECTION AND TREATMENT

- **Specimen collection**
  - Use non-wettable plastic blood collection tubes.
  - Blood is collected in 0.129 M / 0,109M trisodium citrate anticoagulant (using a ratio of 9:1 volumes).
- **Specimen preparation**
  - The test is performed either on citrated whole blood or on platelet rich plasma (PRP).
- **Specimen storage**
  When an anti-aggregant such as **blocking monoclonal antibody** is used (for example, 7E3 MAb) the blood specimen must be treated within 24 hours after collection.
  For any **other anti-aggregant** type, specimen storage time must be experimentally tested.
  Blood is stored at room temperature before testing (18-25°C).

5 PROCEDURE

Note: For good results exercise great care in the pipetting of small reagent volumes by depositing them at the bottom of the test tubes.

All reagents must be at room temperature.
One calibration curve must be run for each series. One series could contain up to 5 samples.

5.1. Reagent Tube SetUp

- Label 5 plastic tubes T1 to T5. Set the tubes in a rack.
- Pipette reagents into tubes as follows:
  Tube T1: Pipette **50 µL** of blood sample and add **150 µL** diluted Reagent 1. Mix.
  Tube T2: **20 µL** Reagent 2a (Negative control),
  Tube T3: **20 µL** Reagent 2b (MAb1),
  Tube T4: **20 µL** Reagent 2c (MAb2),
  Tube T5: **40 µL** Reagent 3 (shake vial well before pipetting).

5.2. Immuno-labeling of samples and control

- In each of tubes T2, T3 and T4 pipette **20 µL** diluted sample; vortex all tubes to mix.
- Incubate all tubes at room temperature for **20 minutes**.

5.3. Fluorescent Staining

- Pipette **20 µL** Reagent 4 in each of tubes T2 to T5; vortex all tubes to mix.
- Incubate all tubes at room temperature for **10 minutes**.
- Pipette **2 mL** diluted Reagent 1 into each of tubes T2 to T5.

Prepared samples may be stored for maximum **2 hours** at 2-8 °C before cytometric analysis.

5.4. Cytometric analysis

Refer to the Operator's Manual of the cytometer for instructions on how to perform cytometric readings.

The selected Mean Fluorescence Intensity (MFI) statistics is the geometric mean, Mn (x) or GeoMean.

Vortex each tube before analysis.

- Calibration analysis: tube T5 (Fig 1)

Create a FS LOG vs SS LOG cytogram. Add a discriminator to minimize the artefactual background. Set up a gate ("A") around the main bead population (Fig. 1a).

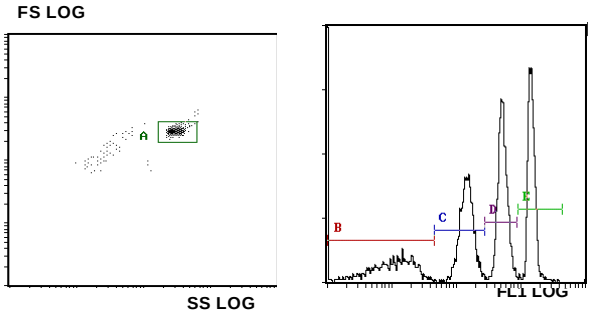
Create a FL_1 LOG gated by the "A" region.

Note the mean fluorescence intensity (MFI) for each of the 4 fluorescence peaks (Fig. 1b : B, C, D and E cursors) corresponding to the 4 calibration beads.

For optimum analysis conditions, the peak of the fourth bead fluorescence intensity (FL1) must be set at the beginning of the fourth decade. To achieve this, adjust the FL1 PMT voltage.

Fig. 1a :
Test calibration cytogram

Fig. 1b :
Cursor settings in gated fluorescence histogram



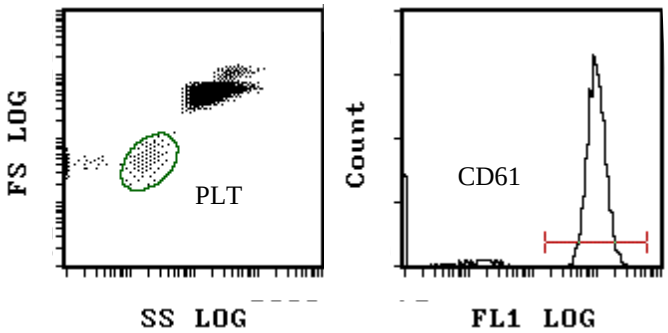
- Sample analysis (Fig 2)

Using the same acquisition procedure, on the FS LOG vs SS LOG histogram (Fig. 2a), platelets are isolated from other whole blood cells by an analysis region " PLT ".

In the corresponding gated fluorescence histogram (Fig. 2b), note the mean fluorescence intensity of each sample.

Fig. 2a :
Whole blood cytogram and platelet region gating

Fig. 2b :
CD61 (GpIIIa) immuno-labeling, cursor settings in PLT gated histogram



6 RESULTS

Depending on the instrument used:
If the MFI values (Mean Fluorescence Intensity) are expressed as linear values, use a log-log graph paper.
If the MFI values are obtained as channel numbers, use a semi-log graph paper.

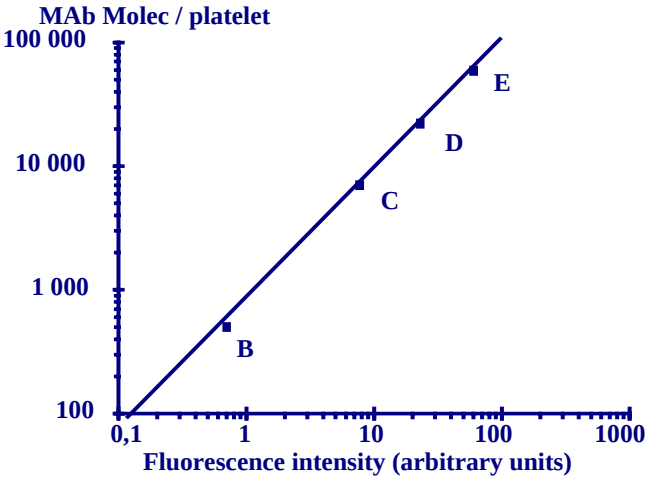
Using a log-log or a semi-log graph paper, plot the MFI calibration values on the abscissa (x-axis) and their corresponding number of monoclonal antibody molecules (as indicated in the assay value insert) on the ordinate (y-axis).

Draw the calibration curve.

Interpolate the MFI values of the tubes T2 to T4 (samples and control) on the calibration curve and read the corresponding numbers of monoclonal antibodies directly off the curve.

Specific quantitative MAb1 and MAb2 values are calculated after subtraction of the negative control measurement.

Example of calibration curve :



Expression of the results :

Antiaggregant type	Total number of GpIIIa sites (*)	Free GpIIIa sites (*)	Number of occupied receptors (*)	Occupancy ratio (%)
Monoclonal antibodies	MAb2	MAb1	MAb2 - MAb1	$\frac{(MAb2 - MAb1)}{MAb2} \times 100$
Peptides and peptido-mimetics	MAb1	MAb2	MAb1 - MAb2	$\frac{(MAb1 - MAb2)}{MAb1} \times 100$

(*) Values are expressed as numbers of MAb molecules bound per platelet.

REFERENCES

1- LEFKOVITS J. et al. Platelet glycoprotein IIb/IIIa receptors in cardiovascular medicine, New Engl. J. Med. 332, pp1553-1559, 1995.

2- HEZARD N. et al. Free and total platelet glycoprotein IIb/IIIa measurement in whole blood by quantitative flow cytometry during and after infusion of c7E3 Fab in patients undergoing PTCA, Thromb.Haemost. 81: pp869-873, 1999.

3- QUINN M. et al. Quantifying GpIIb/IIIa receptor binding using 2 monoclonal antibodies, Circulation 99 :pp2231-2238, 1999.

4- HEZARD N. et al. Use of PFA-100 apparatus to assess platelet function in patients undergoing PTCA during and after infusion of c7E3 Fab in the presence of other antiplatelet agents, Thromb.Haemost. 83: 540-4, 2000.

5- HEZARD N. et al. Unexpected flow cytometric results with two small GpIIb/IIIa blockers: eptifibatide and tirofiban, Thromb.Haemost. 85:561-2, 2001.

BIOCYTEX
140 ch. ARMEE D'AFRIQUE
13010 MARSEILLE
FRANCE
TEL : +33 (0) 4 96 12 20 40
FAX : +33 (0) 4 91 47 24 71

Version February 2002