PLATELET PAIg

Kit for Platelet Associated Immunoglobulin
quantitation

Kit for 10 determinations   **Ref. 7005**

For research use only.

1 METHOD

Dual color flow cytometric analysis of Platelet Associated Immunoglobulins (PAIg).
The number of PAIg is determined by converting the fluorescence intensity into the corresponding number of sites per platelet based on a calibrated bead standard curve.

2 PRINCIPLE

Using the GelSep® tube platelets from the sample are separated from the plasma and washed. Then platelets are incubated with a mixture of two monoclonal antibodies specific of Kappa and Lambda human immunoglobulin light chains. After washing a mixture made of a revelation reagent-FITC and a platelet counter-staining reagent-PE is added to the immuno-labelled sample and to a dedicated dual color calibrator.

3 KIT REAGENTS

- **Reagent 1a :** 1 x 8 mL vial, stabilization buffer, 10 fold concentrated.
- **Reagent 1b :** 1 x 21 mL vial, diluent, 10 fold concentrated.
- **Reagent 2a :** 1 x 200 μL vial, Mab anti human light chains (Kappa + Lambda).
- **Reagent 2b :** 1 x 200 μL vial, negative isotypic control.
- **Reagent 3 :** 1 x 400 μL vial, calibration bead suspension . The beads are coated with increasing and accurately known quantities of mouse IgG. The number of determinants coated on each bead population is indicated in the calibration flyer inserted in the kit.
  A PE bead population, allowing the fluorescence compensation setting, is associated to this suspension.
- **Reagent 4 :** 1 x 600 μL vial, staining reagent, polyclonal antibody anti mouse IgG-FITC + platelet counter-staining reagent-PE (anti CD61-PE).
- **10 GelSep® Tubes** (red top).
- **10 tubes with filter-cap** (blue top).

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.

4 MATERIAL REQUIRED BUT NOT PROVIDED

- Stirring machine type vortex.
- Centrifuge with swinging bucket rotor.
- Timer.
- Cytometer.
- Adjustable pipettes with disposable tips (20 à 200 μL).
- Pipettes (1 and 2 mL).
- Tubes for cytometer.
- Distilled water.

5 REAGENT PREPARATION AND STORAGE

Unopened kit and contents remain stable until expiration date printed on the box label when stored at 2-8°C *.

- **Reagents 1a and 1b **:**
Stability after opening : 2 months at 2-8°C when free contamination.
Prepare a 1:10 dilution with distilled water.
Stability after dilution : 15 days at 2-8°C.
For fractioned use, 8 mL and 21 mL of diluted reagents 1a and 1b are necessary per sample.

- **Reagents 2a, 2b and 4 :** Ready for use.
Stability after opening : 2 months at 2-8°C when free contamination.

- **Reagent 3 :** Ready for use.
Resuspended this reagent by vortexing vigorously for 5 seconds before use.
Stability after opening : 2 months at 2-8°C when free contamination.

Note : * Do not freeze the kit.
** The presence of crystals does not affect the quality of the reagent. In such case, incubate at 37°C until the cristals are completely dissolved.

6 SPECIMEN COLLECTION AND TREATEMENT

- **Sample collection :**
- Use glass blood collection tubes.
- Maintain platelet integrity. Avoid platelet activation during the collection procedure.
- Anticoagulant : EDTA (K3).

- **Sample storage :**
- The blood sample must be treated within **4 hours** after the collection.

7 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 kept at room temperature during the procedure.

One calibration curve must be performed per sample series. A series can contain up to 5 samples.

7.1 Platelet separation

For one separation, 1 GelSep® tube and 1 tube with filter-cap are necessary.
Before immuno-labelling, all resuspension and homogenization of platelet pellet must be realized by gentle aspiration/discharge using a pipet.

7.1.1 sample preparation
1. Centrifuge a minimum volume of **2 mL** of blood sample at **170 g** for **15 minutes** at room temperature (18-25 ºC).
2. In a tube, collect all the PRP without red blood cells and homogenize it by gentle aspiration/discharge using a pipet.
3. Possibility to incubate a volume of PRP with a volume of plasma or serum of known anti-platelet reactivity, 30 minutes at room temperature, to simulate a positive PAIg sample.

7.1.2 Platelet separation from plasma

In the GelSep® tube, add a volume of PRP according to the platelet count:

Platelet Count (on whole blood)	PRP volume to use
From 5.10^9 to < 150.10^9 platelets / L	500 µL
From 150.10^9 to 400.10^9 platelets / L	200 µL

In the GelSep® tube:

1. Gently distribute **2 mL** of **diluted reagent 1a** along the inner sides of the GelSep® tube to avoid the gel resuspension.
2. Transfer with an adjustable pipette, the required volume of homogenized PRP. Incubate **at least 10 minutes** at room temperature.
3. Centrifuge **5 minutes** at **1200 g** and discard the supernatant by inverting down the GelSep® tube.
4. Rinse the GelSep® tube by gently adding **2 mL** of **diluted reagent 1a** along the inner sides of the tube.
5. Discard the supernatant by inverting down the GelSep® tube.
6. Repeat **twice** steps **4 and 5**.

7.1.3 Platelet collection

1. With an adjustable pipette, resuspend the gel containing the platelets and transfer it in the blue filter-cap.
2. Centrifuge **1 minute** at **170 g** the tube with filter cap.
3. Remove the filter cap containing the gel.

Thus treated platelets are separated from plasma, washed and concentrated. Platelets are available in suspension with the diluent and this is recommended to use them **immediately** after separation.

7.2 Sample immuno-labelling

7.2.1 Test tube Setup

Label 3 tubes for cytometry T1 to T3. Set the tubes in a rack.

1. Tube T1 : pipette **20 µL** of **reagent 2a**.
2. Tube T2 : pipette **20 µL** of **reagent 2b**
3. Tube T3 : pipette **40 µL** of **reagent 3** after shaking vigorously this reagent by vortexing.

7.2.2 Immunolabelling

For each tube T1 to T2 :

1. Pipette **20 µL** of filtered platelets.
2. Homogenize using a vortex.
3. Incubate **5 minutes** at room temperature.

7.2.3 Washing

For each tube T1 to T3 :

1. Add **3 mL** of **diluted reagent 1b**.
2. Centrifuge **5 minutes** at **1200 g** and discard the supernatant by inverting down the tube.
3. Repeat **once** steps **1 and 2**.

7.2.4 Revelation

For each tube T1 to T3 :

1. Pipette **20 µL** of **reagent 4.**
2. Homogenize using a vortex.
3. Incubate **5 minutes** at room temperature.
4. Add **1 mL of diluted reagent 1b**.

The test samples may be stored at room temperature for **6 hours** before cytometric analysis.

7.3 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) statistic is the geometric mean, Mn(x) or GeoMean.

Notes : the Beckman Coulter softwares Expo™ 32, CXP and RXP have an option baseline offset. This should be set OFF.

Vortex each tube before analysis**.**

The flow rate must correspond to the **lowest available speed**.

- **Calibration analysis: tube T3 (Figs 1)**
1. Create a FS LOG x SS LOG cytogram.
2. Add a discriminator threshold on FS to eliminate the eventual contaminants (background of the instrument).
3. Draw an analysis region "CAL" around the main singlet bead population (Fig. 1a).
4. Create a FL1 LOG x FL2 LOG cytogram gated by the region "CAL".
5. Adjust the FL1 PMT voltage. The cloud of the fourth bead (FL1) must be set at the beginning of the fourth decade on the FL1 LOG x FL2 LOG cytogram.
6. Also, adjust the FL2 PMT voltage. The cloud of the PE-bead must be set at the beginning of the third decade on the FL1 LOG x FL2 LOG cytogram.
   Do not change these fluorescence settings FL1 LOG, FL2 LOG (PMT v, PMT FL1 and FL2) for the rest of protocol.
7. **Compensation setting for FL2** (Fig. 1b) : on FL1 LOG x FL2 LOG cytogram gated by the region "CAL", set two regions ("B" and "C") on the clouds corresponding to the lowest and the highest FL1+ calibration beads, respectively. Adjust compensation settings to get similar MFI FL2 value in regions "B" and "C".
8. **Compensation setting for FL1** (Fig. 1b) : on FL1 LOG x FL2 LOG cytogram gated by the region "CAL", set two regions ("B" and "D") on the clouds corresponding to the lowest and the FL2+ calibration beads, respectively. Adjust compensation settings to get similar MFI FL1 value in regions "B" and "D".
9. Create a FL1 LOG histogram gated by the region "CAL".
10. Note the mean of fluorescence intensity (MFI) for each of the 4 fluorescence peaks (Fig. 1c : A, B, C and D cursors) corresponding to the 4 calibration beads. **For a correct analysis, a minimum of 10,000 beads must be gated on the window "CAL".**
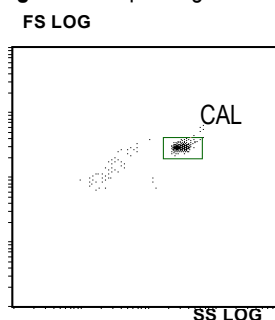
Fig. 1a : Setup of region "CAL"
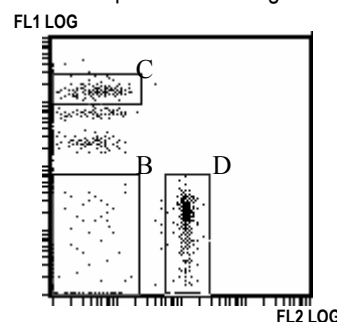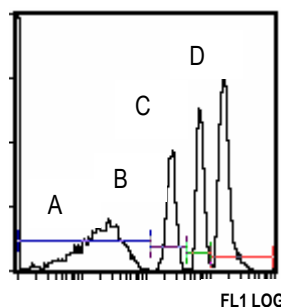
Fig.1b Fluorescence compensation setting


Fig. 1c : Calibrator

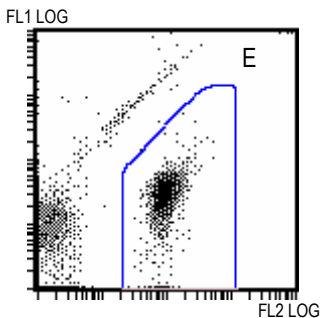**Note : cursor A has to include the first channel.**

- **Sample Analysis : tubes T1 and T2 (Figs 2)**
1. Analyze tube T1 at first.
2. On the FS LOG x SS LOG cytogram, enlarge region "PLT" as shown (Fig. 2a) to analyze all events.
3. On the FL1 LOG x FL2 LOG cytogram gated by the region " PLT", set up a region "E" on the FL2 positive platelet population, avoiding to include non specific FL1/FL2 positive comet events (Fig. 2b).
4. Create a FL1 LOG histogram gated by the region "E".
5. Note the mean of fluorescence intensity on the whole histogram (cursor F) of tube T1 (Fig. 2c). **Analyse at least 10,000 events in region "E".**
6. **Without modifying region "E",** analyze tube T2 and note the mean fluorescence intensity on the whole histogram.
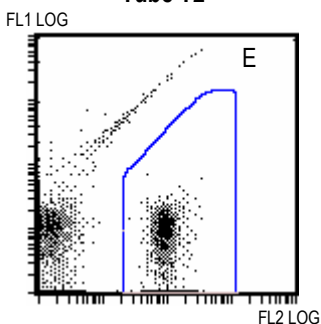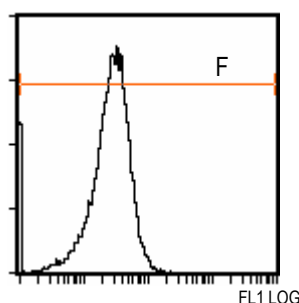
Fig. 2a : Platelets
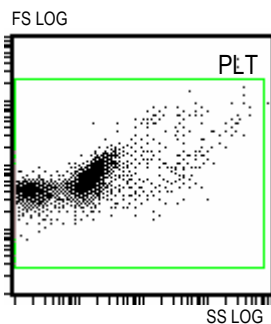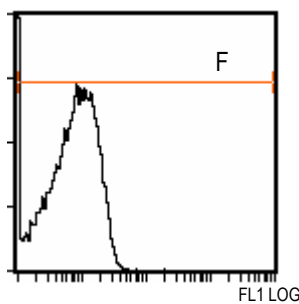

Fig. 2b : Immuno labelling Region setting

Fig. 2c : Immuno labelling Cursor setting

Tube T1

Tube T2

<u>Note</u> **: cursor F has to include the first channel.**

8 RESULT INTERPRETATION

Computer or graphic data analysis.

8.1 Computer data analysis
The result treatment is easily performed using a calculation template available upon request from the BioCytex technical department.

8.2 Graphic data analysis
Plot the LOG_{10} of MFI calibration values (tube T3) on the abscissa (x-axis) and the corresponding LOG_{10} number of monoclonal antibody molecules (as indicated in calibration flyer) on the ordinate (y-axis).

Draw the optimal calibration curve of the type $LOG10(ABC) = a \times LOG10(MFI) + b$.

Interpolate the LOG_{10} of MFI values of the tubes T1 and T2 on the calibration curve and read off directly their corresponding molecule numbers. (ABC : Antibody Binding Capacity)

PAIg specific quantitative values (sABC : specific Antibody Binding Capacity) are calculated as follow :

$T1_{sABC} = T1_{ABC} - T2_{ABC}$

Example of calibration curve (for a Beckman-Coulter instrument type Epics XL) :
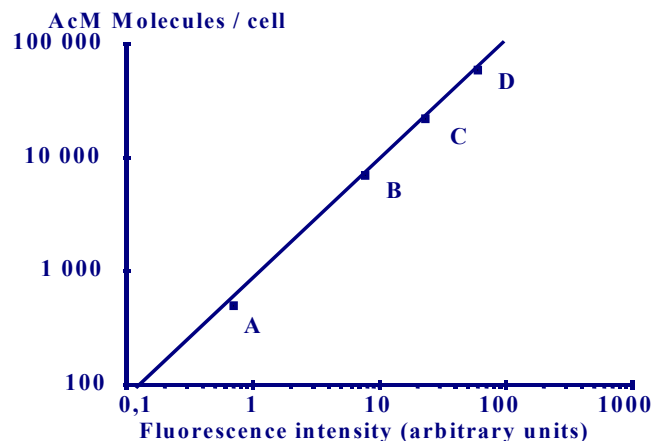
9 EXPECTED VALUES – INTERPRETATION
The specific quantitative values are calculated and compared to the expected values, defined on a normal population.

We recommend that each laboratory define its own expected values, from a local population of healthy donors with similar pre-analytical values. [1]

Values determined on samples drown from normal adult donors, the cytometric analysis was performed on Coulter EPICS® XL-MCL cytometer. Blood samples (n=19) have been analyzed at Day 0, Day 1 and Day 2 after collection (only 13 samples were tested at Day 2).

PAIg	Day 0	Day 1	Day 2
Mean	**1358**	**1575**	**1878**
SD	449	514	540
n	19	19	13

Results expressed on sABC

10 PERFROMANCES

PLATELET PAIg has been validated against GTI PAKAUTO kit （ $C \in$ ）
on 19 normal samples and 10 pathological samples.

PLATELET PAIg features the following performances :

Specificity : 90 %
Sensitivity : 100 %
Negative Predictive Value : 100 %
Positive Predictive Value : 81.8 %

11 LIMITATION

PLATELET PAIg cannot be used :
- on blood samples presenting Reopro or Abciximab.
Indeed, Abciximab binds to GpIIb/IIIa glycoprotein of human platelets and
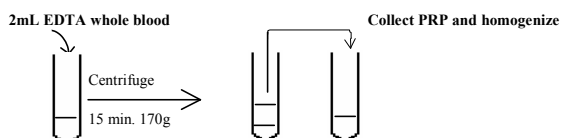the reagent 2a recognises the Reopro at the platelet surface.
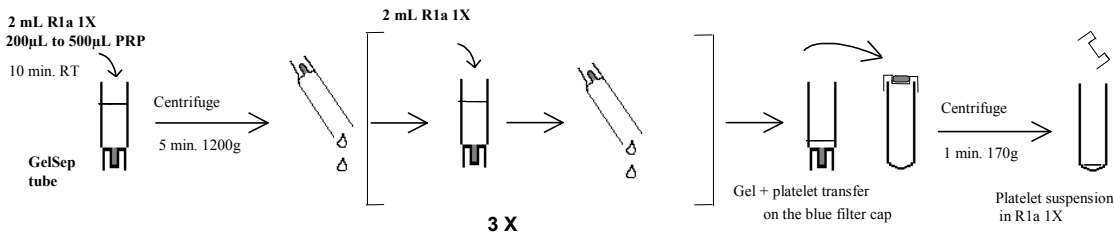- on hemolyzed or hyperlipemic blood samples.

12 BIBLIOGRAPHY

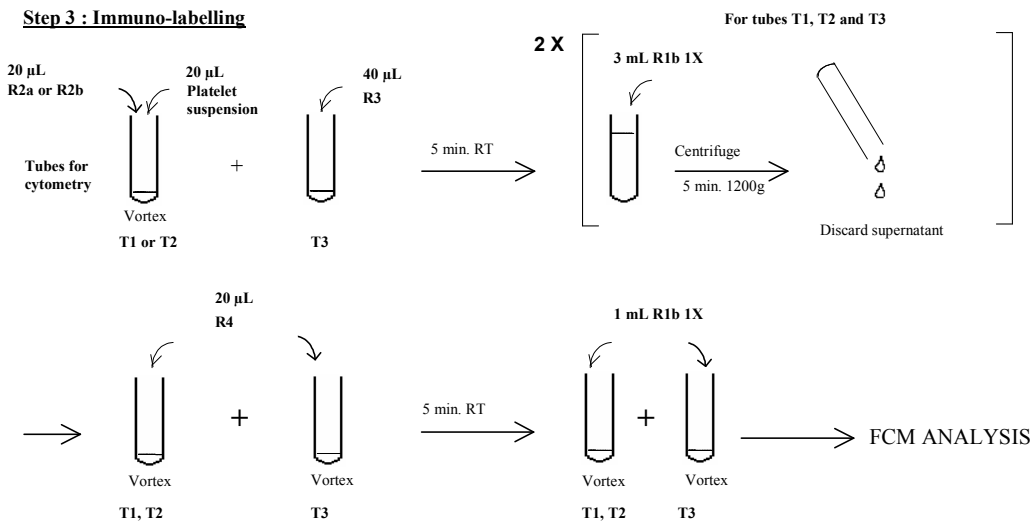[1] Hagenstrom H. *et al.*, Thromb Haemost 2000, 84 : 779-83.

13 PROTOCOL SUMMARY

Step 1 : PRP preparation

Step 2 : Platelet separation

Step 3 : Immuno-labelling

FCM ANALYSIS