1 INTRODUCTION
Platelets are involved in the repair of vessel wall after injury. Platelets aggregation and adhesion to the vessel wall are mediated by cell surface glycoproteins.

Two well-known bleeding disorders involve either a reduced expression or a functional defect in one of the major glycoprotein complex, i.e.:
- Glanzmann Thrombasthenia, which can affect GpIib/Illa expression, and which severity depends on the GpIib/Illa expression level.
- Bernard-Soulier Syndrome, a quantitative or qualitative defect of GpIib/IX/V complex.

GMP140 (P-selectin, PADGEM) levels point out platelet activation state: it is low expressed on resting platelets, and its secretion occurs after activation. GMP140 expression after in vitro activation is a marker of platelet reactivity. Some platelet α granule deficiencies are characterized by a reduced GMP140 secretion after platelet activation.

PLT Gp/Receptors kit allows precise quantitation of GpIIb, GpIb complex and GMP140 glycoproteins on platelet surface, at the resting state and after TRAP (Thrombin Receptor Agonist Peptide) activation.

This kit detects constitutive thrombopathies involving quantitative glycoprotein defects and platelet reactivity defects.

2 PRINCIPLE
The sample is diluted in the presence or absence of TRAP. After dilution, this sample is incubated with different mouse monoclonal antibodies (MAb) directed against human GpIIb, GpIb and GMP140 and a negative isotypic control.

The mean fluorescence intensity (MFI) is measured with a flow cytometer after addition of a staining reagent to the sample and calibrator tubes. Through this calibrator (coated with defined increasing numbers of MAb molecules), the MFI is converted into the absolute number of MAb molecules bound per platelet (ABC).

The results are expressed in sABC (specific ABC equivalent) in this system to the number of Gp molecules per platelet on the surface.

3 KIT REAGENTS
- Reagent 1: 1 vial, 15 mL, diluent, 10 fold concentrated.
- Reagent 2a: 1 vial, 200 µL, negative isotypic control (mouse monoclonal antibody, IgG).
- Reagent 2b: 1 vial, 200 µL, anti GMP140 MAb (CD62P).
- Reagent 2c: 1 vial, 200 µL, anti GpIIb MAb (CD41).
- Reagent 2d: 1 vial, 200 µL, anti GpIb MAb (CD42b).
- Reagent 3: 1 vial, 200 µL, calibrated bead suspension. The beads are coated with increasing and accurately known quantities of mouse IgG.
- Reagent 4: 1 vial, 0.9 mL, staining reagent, polyclonal anti mouse IgG-FITC.
- Reagent 5: 1 vial, TRAP, freeze-dried, (60 nmols / vial).

4 MATERIAL REQUIRED BUT NOT PROVIDED
- Stirring machine type vortex.
- Timer.
- Cytometer.
- Haemolysis tubes for cytometer.
- Adjustable pipettes with disposable tips (20 µL to 1 mL).
- Pipettes (2 to 25 mL).
- Distilled water, deionized water or water for injectable solution.

5 REAGENT PREPARATION AND STORAGE
Intact kits and contents remain stable until the expiration date printed 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 series to be tested.
  Stability after dilution: 15 days at 2-8 °C.

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

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

- Reagent 5
  Open the vial carefully.
  Reconstitute with 500 µL of diluted Reagent 1.
  Stability after reconstitution: 1 month at 2-8 °C.

Notes:
* 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 crystals are completely dissolved.

6 WARNING
- Follow the standard good laboratory practices.
- Follow the appropriate reglementation for waste disposal.
- Blood must be considered as potentially infectious.
- 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.

7 SPECIMEN COLLECTION AND TREATMENT
- Sample collection:
  - Use non-wettable blood collection tubes.
  - Maintain platelet integrity. Avoid platelet activation during the collection procedure (shaking, temperature variations).
  - Anticoagulant: trisodium citrate 0.109 M or 0.129 M (using 9 volumes blood, 1 volume citrate).

- Sample storage:
  - Blood is stored at room temperature before testing (18-25°C).
  - Let the sample stabilize 1 hour before testing.
  - Then, the blood sample must be treated in the following 5 hours.
  - Do not freeze the sample.

8 PROCEDURE
Note: for good results exercise great care in the pipetting of small reagent volumes (20 µL) by depositing them at the bottom of the test tubes.
All reagents must be kept at room temperature during the procedure.

8.1. Test Tube Setup
- Label 11 plastic tubes T1 to T11. Set the tubes in a rack.

Distribute reagents into tubes as follows:
- In tube T1: pipette 50 µL of diluted Reagent 1.
- In tube T2: pipette 50 µL of reconstituted Reagent 5.
- In each tube T3 and T7: pipette 20 µL of Reagent 2a.
- In each tube T4 and T8: pipette 20 µL of Reagent 2b.
- In each tube T5 and T9: pipette 20 µL of Reagent 2c.
- In each tube T6 and T10: pipette 20 µL of Reagent 2d.
- In tube T11: pipette 40 µL of Reagent 3, after shaking vigorously this reagent by vortexing.

8.2. Platelet activation
To each tube T1 and T2:
- Pipette 50 µL of whole blood.
- Homogenize using a vortex for approx. 1-2 sec.
- Incubate the tubes for 5 minutes at room temperature.
- Add 500 µL of diluted Reagent 1.
- Homogenize the tubes using a vortex for approx. 1-2 sec.
8.3. Samples immuno-labelling
For each tube T3 to T6:
- Add 20 µL of the diluted sample from tube T1.
- Homogenize the tubes using a vortex for approx. 1-2 sec.

For each tube T7 to T10:
- Add 20 µL of activated sample from tube T2.
- Homogenize the tubes using a vortex for approx. 1-2 sec.
- Incubate the tubes T3 to T10 for 10 minutes at room temperature.

8.4. Fluorescent staining
For each tube T3 to T11:
- Add 20 µL of Reagent 4.
- Homogenize using a vortex for approx. 1-2 sec.
- Incubate at room temperature for 10 minutes.
- Add 2 mL of diluted Reagent 1.
- Homogenize the tubes using a vortex for approx. 1-2 sec and store immediately at 2-8°C until the cytometric analysis.

The test samples may be stored for 4 hours at 2-8°C before cytometric analysis.

8.5. 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.

- Calibration analysis: tube T11 (Figs. 1)
  Create a FS LOG vs SS LOG cytogram. Add a discriminator on FSLog to minimize the artefactual background. Set up a gate “CAL” around the main single bead population (Fig. 1a).

  Create a FL1 LOG histogram gated by the “CAL” window. Note the MFI for each of the 3 fluorescence peaks (Fig. 1b: A, B and C cursors) corresponding to the 3 calibration beads. The “A” cursor must include the first channel.

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

  The “A” cursor must include the first channel.

  ```
  Fig.1a: Test calibration cytogram
  Fig.1b: Cursor setting in gated fluorescence histogram
  ```

- Sample analysis: Tube T3 to T10 (Figs. 2)
  Do not change the acquisition procedure for FL1 (PMTv).
  On the FS LOG vs SS LOG cytogram, platelets are isolated from the other whole blood cells by an analysis window “PLT” (Fig. 2a). Check that the discriminator setting does not cut the platelet cloud.
  Analyse at least 3,000 events on the window “PLT”.
  In the corresponding gated fluorescence histogram, note the MFI of each assay (Figs. 2b and 2c). A good analysis allows to obtain at least 50% of positive cells in the window “PLT”.

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  Fig.2a: Setting of the “PLT” window
  Fig.2b: CD62P Immuno-labelling: Cursor setting
  ```

  For weakly expressed markers (isotypic negative control, GMP 140 basal state), note the MFI corresponding to the whole platelet population (Fig. 2b). The cursor must absolutely take the first channel into account.

  ```
  Fig.2c: CD41 immuno-labelling. Cursor setting
  ```

  For clearly expressed markers (GMP140 activated state, GpIIb and GpIbα), note only the MFI corresponding to the positive peak of interest (Fig. 2c).

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  Example:
  sABC CD41 = ABC CD41 (tube T5) – ABC isotypic negative control (tube T3).
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  Example of a calibration curve (for a Beckman Coulter instrument type Epics XL):
  ```

8.6 Result analysis
Computer or graphic data analysis. Plot the LOG10 of the MFI calibration values for the 3 calibration beads (tube T11) on the abscissa (x-axis), and their corresponding LOG10 number of monoclonal antibody molecules as indicated on the calibration flyer, on the ordinate (y-axis). Draw the optimal calibration curve of the type LOG10(ABC) = a x LOG10(MFI) + b

Interpolate the LOG10 of the MFI values of tubes T3 to T10 on the calibration curve and read directly their corresponding numbers of bound Mab molecules (ABC: Antibody Binding Capacity).

The specific quantitative values (sABC: specific Antibody Binding Capacity) are determined by subtracting the negative isotypic control ABC value as follows:

Example:

```n
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9 RESULT 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 covering the same age range as the tested patients.

Values determined on 40 samples drawn from normal adult donors.

<table>
<thead>
<tr>
<th>Basal state</th>
<th>Activated state</th>
</tr>
</thead>
<tbody>
<tr>
<td>GMP 140 (CD62P)</td>
<td>&lt; 1,000</td>
</tr>
<tr>
<td>GpIb (CD42a)</td>
<td>51,000 +/- 14,000</td>
</tr>
<tr>
<td>GpIb (CD42b)</td>
<td>38,000 +/- 11,000</td>
</tr>
</tbody>
</table>

These values are not suitable for children (age ranged from 0 to 18 years). Platelet glycoproteins are differently expressed on children population, and their expression depends on age (8).

Example of values on Glanzmann thrombasthenia syndromes (2) (sABC):

<table>
<thead>
<tr>
<th>Homozygous 1</th>
<th>Resting state</th>
<th>Activated state</th>
</tr>
</thead>
<tbody>
<tr>
<td>GMP 140 (CD62P)</td>
<td>&lt; 1,000</td>
<td>6,000</td>
</tr>
<tr>
<td>GpIb (CD42a)</td>
<td>1,300</td>
<td>2,000</td>
</tr>
<tr>
<td>GpIb (CD42b)</td>
<td>41,000</td>
<td>32,000</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Heterozygous 2</th>
<th>Resting state</th>
<th>Activated state</th>
</tr>
</thead>
<tbody>
<tr>
<td>GMP 140</td>
<td>&lt; 1,000</td>
<td>11,600</td>
</tr>
<tr>
<td>GpIb</td>
<td>28,000</td>
<td>51,000</td>
</tr>
<tr>
<td>GpIb</td>
<td>40,000</td>
<td>33,000</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Heterozygous 3</th>
<th>Resting state</th>
<th>Activated state</th>
</tr>
</thead>
<tbody>
<tr>
<td>GMP 140</td>
<td>&lt; 1,000</td>
<td>9,700</td>
</tr>
<tr>
<td>GpIb</td>
<td>25,000</td>
<td>49,000</td>
</tr>
<tr>
<td>GpIb</td>
<td>31,000</td>
<td>13,000</td>
</tr>
</tbody>
</table>

Example of values on a Fechtner syndrom (2) (sABC):

<table>
<thead>
<tr>
<th>GMP 140 (CD62P)</th>
<th>Resting state</th>
<th>Activated state</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 1,000</td>
<td>19,200</td>
<td></td>
</tr>
<tr>
<td>GpIb (CD41a)</td>
<td>137,000</td>
<td>204,000</td>
</tr>
<tr>
<td>GpIb (CD42b)</td>
<td>111,000</td>
<td>43,000</td>
</tr>
</tbody>
</table>

10 PERFORMANCES

PLT Gp/Receptors kit has been validated on Becton-Dickinson instruments type FACS Calibur and Beckman-Coulter type XL and XL MCL (System II™ software).

- **Test specificity:**
  The PLT Gp/Receptors kit has been tested on constitutive thrombopathic samples. The results correlate perfectly with clinical observations and genetic tests (2) (3).

- **Reagents specificity:**
  Reagent 2a monoclonal antibody is not directed against a human epitope. Reagent 2b antibody is specifically directed against GMP140 (8), Reagent 2c antibody has been clustered as CD41 (Leucocyte Typing V) (4), Reagent 2d antibody has been clustered as CD42b (Leucocyte Typing V) (8).

- **Repeatability:**
  Normal samples (n=2) are tested 5 times with the same kit (sABC):

<table>
<thead>
<tr>
<th>GMP140</th>
<th>Resting state</th>
<th>Activated state</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 1,000</td>
<td>12,580</td>
<td>860</td>
</tr>
<tr>
<td>GpIb</td>
<td>1,970</td>
<td>61,700</td>
</tr>
<tr>
<td>GpIb</td>
<td>1,020</td>
<td>23,210</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>GMP140</th>
<th>Resting state</th>
<th>Activated state</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 1,000</td>
<td>15,350</td>
<td>910</td>
</tr>
<tr>
<td>GpIb</td>
<td>960</td>
<td>94,570</td>
</tr>
<tr>
<td>GpIb</td>
<td>1,190</td>
<td>22,920</td>
</tr>
</tbody>
</table>

- **Inter lot reproducibility (sABC):**
  One sample is tested twice with 2 different batches:

<table>
<thead>
<tr>
<th>GMP140 (CD62P)</th>
<th>Resting state</th>
<th>Activated state</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch 1</td>
<td>&lt; 1,000</td>
<td>13,860</td>
</tr>
<tr>
<td>Batch 2</td>
<td>&lt; 1,000</td>
<td>14,630</td>
</tr>
<tr>
<td>GpIb (CD41a)</td>
<td>56,540</td>
<td>102,620</td>
</tr>
<tr>
<td>GpIb (CD42b)</td>
<td>64,570</td>
<td>86,470</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>GMP140 (CD62P)</th>
<th>Resting state</th>
<th>Activated state</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch 1</td>
<td>36,390</td>
<td>20,530</td>
</tr>
<tr>
<td>Batch 2</td>
<td>40,060</td>
<td>21,350</td>
</tr>
</tbody>
</table>

11 LIMITATIONS

PLT Gp/Receptors kit can only be used on samples meeting the following requirements:
- sample not hemolyzed.
- absence of platelet activation. At the basal state the CD62P expression should be lower than 1000 sABC.
- absence of GpIIa antagonist therapy. Confirm with the PLATELET GpIIb/IIIa Occupancy kit (BioCytex, Ref. 7001), in case of doubt.
- absence of anti platelet auto- or allo-antibodies.

12 LIABILITY

The in vitro diagnostic use is only valid within the strict application of the package insert. Any modification of the protocol can influence the result of the tests. Do not switch vials from different lots.

In these cases no contestation or replacement of the product will be accepted.

13 REFERENCES

6. PARMENTIER S. et al., "Inhibition of Platelet functions by a monoclonal antibody (Lyp20) directed against a granule membrane glycoprotein (GMP140 / PADGEM)". Blood (1991), 77, 1734-1739.

14 SYMBOLS

<table>
<thead>
<tr>
<th>REF</th>
<th>Catalogue number</th>
<th>Use by</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVD</td>
<td>In vitro Diagnostic Medical Device</td>
<td>Contains sufficient for n° tests</td>
</tr>
<tr>
<td>Temperature limitation</td>
<td>LOT</td>
<td>Batch code</td>
</tr>
</tbody>
</table>

**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 December 2018