PLT Gp/Receptors
Diagnosis of quantitative platelet glycoprotein abnormalities
For In Vitro Diagnostic Use

Out of Europe, this product is dedicated for research use only.

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/IIIa expression, and which severity depends on the GpIIb/IIIa expression level (2).
- Bernard-Soulier Syndrome, a quantitative or qualitative defect of GpIb/IX/V complex (1)(4)(7).

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 quantification of GpIIb, GpIbα 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, GpIbα 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 GpIbα MAb (CD42b).
- Reagent 3 : 1 vial, 200 µL, calibrate 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 on the calibration flyer inserted in the kit. These values may vary from lot to lot.
- Reagent 4 : 1 vial, 0.9 mL, staining reagent, polyclonal anti mouse IgG-FITC.
- Reagent 5 : 1 vial, TRAP, freeze-dried, (60 nmoles / 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
  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 regulationment 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-wettatable 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.
- Incube 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 the tubes at 2-8°C until the cytometric analysis.

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.

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.

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

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.

8.6. Result analysis
Computer or graphic data analysis. Plot the MFI calibration values for the 3 calibration beads (tube T11) on the abscissa (x-axis), and their corresponding log(MFI) + b.

For weakly expressed markers (isotypic negative control, GMP 140 basal state), note only the MFI corresponding to the positive peak of interest (Fig. 2c).

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
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• 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 the tubes at 2-8°C until the cytometric analysis.

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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.

These values are not suitable for children (age ranged from 0 to 18 years).

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 (CD41a)</td>
<td>1,300</td>
<td>2,000</td>
</tr>
<tr>
<td>GpIIb (CD41b)</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 (CD41a)</td>
<td>28,000</td>
<td>51,000</td>
</tr>
<tr>
<td>GpIIb (CD41b)</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 (CD41a)</td>
<td>25,000</td>
<td>49,000</td>
</tr>
<tr>
<td>GpIIb (CD41b)</td>
<td>31,000</td>
<td>13,000</td>
</tr>
</tbody>
</table>

Example of values on a Fechtner syndrome (\(^2\)) (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>GpIIb (CD41b)</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 FACSCalibur 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 clusterized as CD41 (Leucocyte Typing V) (\(^8\)).
Reagent 2d antibody has been clusterized as CD42b (Leucocyte Typing V) (\(^8\)).

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

<table>
<thead>
<tr>
<th>Ech. A</th>
<th>GMP140</th>
<th>Resting state</th>
<th>Mean</th>
<th>SD</th>
<th>CV</th>
<th>Activated state</th>
<th>Mean</th>
<th>SD</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt; 1,000</td>
<td>12.580</td>
<td>860</td>
<td>6.8%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GpIb</td>
<td>1,970</td>
<td>3.1%</td>
<td>110.700</td>
<td>6.080</td>
<td>5.5%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GpIIb</td>
<td>44.205</td>
<td>1,020</td>
<td>2.3%</td>
<td>23.210</td>
<td>880</td>
<td>3.8%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ech. B</th>
<th>GMP140</th>
<th>Resting state</th>
<th>Mean</th>
<th>SD</th>
<th>CV</th>
<th>Activated state</th>
<th>Mean</th>
<th>SD</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt; 1,000</td>
<td>15,350</td>
<td>910</td>
<td>5.9%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GpIb</td>
<td>960</td>
<td>1.7%</td>
<td>94.570</td>
<td>6.780</td>
<td>7.2%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GpIIb</td>
<td>40.810</td>
<td>1,190</td>
<td>2.9%</td>
<td>22.920</td>
<td>1,050</td>
<td>4.6%</td>
<td></td>
<td></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>&lt; 1,000</td>
</tr>
<tr>
<td>Batch 2</td>
<td>&lt; 1,000</td>
<td>&lt; 1,000</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>GpIb (CD41)</th>
<th>Resting state</th>
<th>Activated state</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch 1</td>
<td>56.540</td>
<td>55.470</td>
</tr>
<tr>
<td>Batch 2</td>
<td>54,570</td>
<td>55,230</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>GpIIb (CD42b)</th>
<th>Resting state</th>
<th>Activated state</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch 1</td>
<td>36,390</td>
<td>37,560</td>
</tr>
<tr>
<td>Batch 2</td>
<td>40,060</td>
<td>40,080</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 / PAGEM)". Blood (1991), 77, 1734-1739.