PLT Gp/Receptors

Diagnosis of quantitative platelet glycoprotein abnormalities

For In Vitro Diagnostic Use

Kit for 5 determinations

CE

Ref. 7004

BioCytex

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 quantitation 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 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. 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**

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 the tubes 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 FL_1 LOG histogram gated by the "CAL" window. Note the MFI for each of 3 fluorescence peaks (Fig. 1b: A, B and C cursors) corresponding to the 3 calibration beads.

For a correct analysis, at least 8,000 beads must be gated on the window "CAL".

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

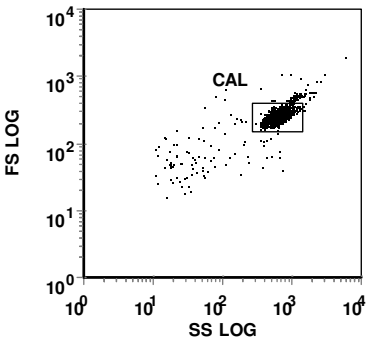
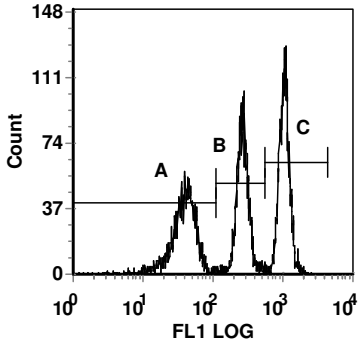


Fig.1b: Cursor setting in gated fluorescence histogram



• 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".**

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.2a: Setting of the "PLT" window

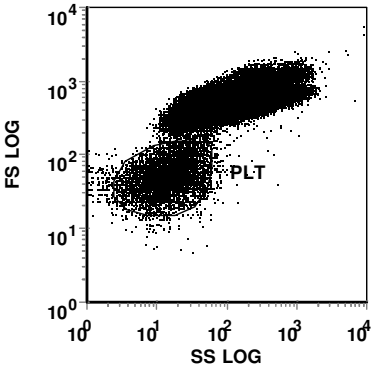
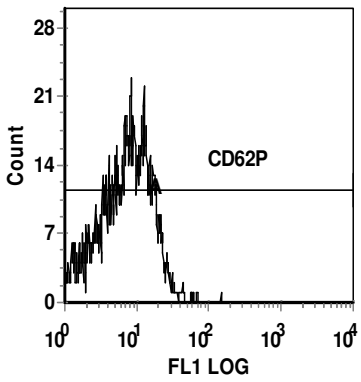
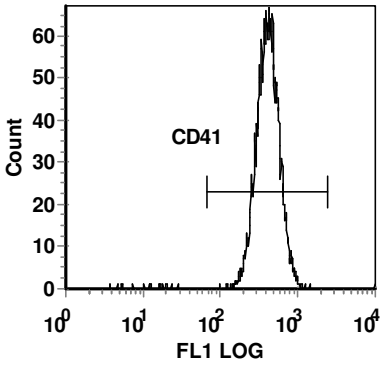


Fig.2b: CD62P Immuno-labelling: Cursor setting



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

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



8.6 Result analysis

Computer or graphic data analysis.

Plot the LOG_{10} of the MFI calibration values for the 3 calibration beads (tube T11) on the abscissa (x-axis), and their corresponding LOG_{10} number of monoclonal antibody molecules as indicated on the calibration flyer, on the ordinate (y-axis).

Draw the optimal calibration curve of the type $LOG_{10}(ABC) = a \times LOG_{10}(MFI) + b$

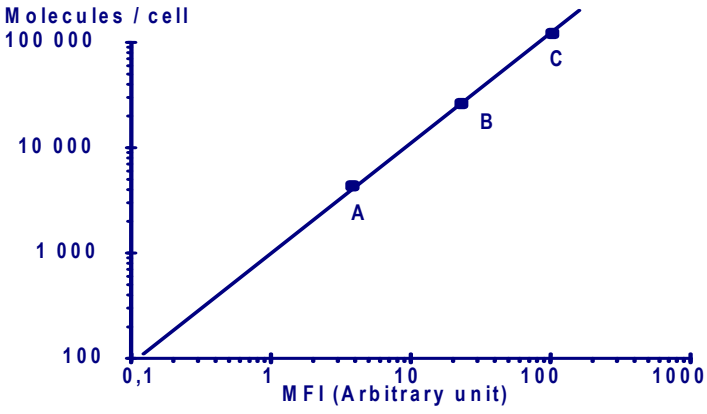
Interpolate the LOG_{10} of the MFI values of tubes T3 to T10 on the calibration curve and read directly their corresponding numbers of bound Mab molecules (ABC: Antibody Binding Capacity).

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

Example:

$sABC_{CD41} = ABC_{CD41}$ (tube T5) – $ABC_{isotypic\ negative\ control}$ (tube T3).

Example of a calibration curve (for a Beckman Coulter instrument type Epics XL):



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 drown from normal adult donors.

	Basal state	***Activated state***
GMP 140 (CD62P)	< 1,000	≥ 1,000
GpIIb (CD41a)	51,000 +/- 14,000	85,000 +/- 27,000
GpIbα (CD42b)	38,000 +/- 11,000	19,000 +/- 10,000

Results expressed on sABC

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 [5].

Example of values on Glanzmann thrombasthenia syndroms [2] (sABC):

		Resting state	***Activated state***
Homozygous 1	GMP 140 (CD62P)	< 1,000	6,000
	GpIIb (CD41a)	1,300	2,000
	GpIb (CD42b)	41,000	32,000
Heterozygous 2	GMP 140	< 1,000	11,600
	GpIIb	28,000	51,000
	GpIb	40,000	33,000
Heterozygous 3	GMP 140	< 1,000	9,700
	GpIIb	25,000	49,000
	GpIb	31,000	13,000

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

	Resting state	***Activated state***
GMP 140 (CD62P)	< 1,000	19,200
GpIIb (CD41a)	137,000	204,000
GpIb (CD42b)	111,000	43,000

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 [6].
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):

		Resting state			***Activated state***		
		Mean	**SD**	**CV**	**Mean**	**SD**	**CV**
Ech. A	**GMP140**	< 1,000	-	**-**	12,580	860	***6.8 %***
	GpIIb	64,350	1,970	***3.1 %***	110,700	6,080	***5.5 %***
	GpIb	44,205	1,020	***2.3 %***	23,210	880	***3.8 %***
Ech. B	**GMP140**	< 1,000	-	**-**	15,350	910	***5.9 %***
	GpIIb	55,720	960	***1.7 %***	94,570	6,780	***7.2 %***
	GpIb	40,810	1,190	***2.9 %***	22,920	1,050	***4.6 %***

- **Inter lot reproducibility (sABC):**

One sample is tested twice with 2 different batches:

		Resting state		***Activated state***	
GMP140 (CD62P)	**Batch 1**	< 1,000	< 1,000	13,860	14,230
	Batch 2	< 1,000	< 1,000	14,630	15,010
GpIIb (CD41)	**Batch 1**	56,540	55,470	102,820	96,550
	Batch 2	54,570	55,230	86,470	91,920
GpIb (CD42b)	**Batch 1**	36,390	37,560	20,530	22,900
	Batch 2	40,060	40,080	21,350	22,570

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 GpIIIa 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 accept.

13 REFERENCES

1. MORAN N. *et al*., "Surface expression of glycoprotein Ib alpha is dependent on GpIb beta: evidence from a novel mutation causing Bernard-Soulier syndrome". Blood (2000), 96, 532-539.
2. SCHLEGEL N. *et al.,* "The interest of quantitative flow cytometry kits for the identification and characterization of inherited platelet disorders", Blood (1998) Suppl. 1, 92, Abst. 3367.
3. SCHLEGEL N. *et al*., "Is X-linked familial thrombocytopenia (XLT) a pure platelet variant of Wiskott Aldrich Syndrome (WAS)." Thromb. Haemost. (1999) Suppl. 797, Abst. 2524.
4. BUNESCU A. *et al*., "Partial expression of GpIb measured by flow cytometry in two patients with Bernard-Soulier syndrome" Thromb. Res. (1994), 76, 441-450.
5. HEZARD N. *et al*., "Unexpected persistence of platelet hyporeactivity beyond the neonatal period: a flow cytometric study in neonates, infants and older children". Thromb. Haemost. (2003), 90, 116-23.
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.
7. DROUIN J. *et al*. "Compound heterozygosity for a novel nine-nucleotide deletion and the Asn45Ser Missense Mutation in the Glycoprotein IX in a patient with Bernard-Soulier Syndrome" Am. J. of Hematol. (2005) 78: 41-48.
8. SCHLOSSMAN, S.F. *et al. -Eds,* Leucocyte Typing V, Oxford University Press (1995).

14 SYMBOLS

REF	*Catalogue number*		*Use by*
IVD	*In vitro Diagnostic Medical Device*	Σ	*Contains sufficient for n" tests*
	Temperature limitation	LOT	*Batch code*
	Manufacturer		

BIOCYTEX
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