

PLT HPA-1

Determination of the platelet HPA-1 polymorphism by flow cytometry

For In Vitro Diagnostic Use

Kit for 10 determinations



Ref. 7003



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

1 INTRODUCTION

Platelet glycoprotein polymorphism constitutes a target for allo-immunisation. Now, 16 polymorphisms have been identified on the HPA system (Human Platelet Antigens) (7) (8).

Anti HPA alloantibodies are responsible for clinical syndromes and transfusion related conditions such as fetomaternal alloimmune thrombocytopenia (FMAIT) or neonatal alloimmune thrombocytopenia (NAIT), post-transfusion purpura (PTP) and platelet transfusion refractoriness (PTR).

In Caucasians, the HPA-1a antigen is the most commonly involved in these pathologies. This is due to a mutation (176T>C) located on the gene ITGB3 leading to the amino-acid substitution L33P on the glycoprotein GpIIIa.

The Caucasian HPA-1 genotype frequencies are estimated as followed :

Genotypes	HPA-1a/1a	HPA-1a/1b	HPA-1b/1b
Frequency (%) (5)	72.0	26.8	1.2

2 PRINCIPLE

The diluted sample is incubated separately with two monoclonal antibodies (MAbs) : MAb1 has a low affinity for the HPA-1b allele (6) and MAb2 binds to the GpIIIa independently of the HPA-1 polymorphism.

A staining reagent is added to the sample and to an appropriated calibrator. Due to this calibrator the number of MAb molecules bound to the GpIIIa may be quantitated by flow cytometric analysis. Quantitating the platelet binding capacity of both anti GpIIIa MAbs allows to calculate the HPA-1 ratio R (Cf. § 9) and to determine the HPA-1 genotype of the sample.

3 KIT REAGENTS

- **Reagent 1** : 1 x 15 mL vial, diluent, 10 fold concentrated.
- **Reagent 2a** : 1 x 200 µL vial, MAb anti GpIIIa, **MAb1**.
- **Reagent 2b** : 1 x 200 µL vial, MAb anti GpIIIa, **MAb2**.
- **Reagent 3** : 1 x 400 µL vial, mixture of 3 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 x 600 µL vial, staining reagent, polyclonal anti-mouse IgG-FITC.

4 MATERIAL REQUIRED BUT NOT PROVIDED

- Stirring machine type vortex.
- Timer.
- Cytometer.
- Centrifuge.
- Haemolysis tubes for cytometer.
- Adjustable pipettes with disposable tips (10 µL to 1 mL).
- Pipettes (1 or 2 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 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.

Notes :

* Do not freeze the kit.

** The presence of crystals does not affect the quality of the reagent. In such a 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, heat shock).
- Anticoagulant : **trisodium citrate 0.109 M or 0.129 M** (using 9 volumes blood, 1 volume citrate).

- **Sample storage :**

- The blood sample must be treated within **24 hours** after collection.
- Blood is stored at room temperature before testing (18-25°C).
- 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 Reagent tube Setup

- Label 4 plastic tubes T1 to T4. Set them in a rack.
- Perform the following pipetting steps :
Tube T1 : pipette **150 µL** of diluted Reagent 1.
Tube T2 : pipette **20 µL** of Reagent 2a.
Tube T3 : pipette **20 µL** of Reagent 2b.
Tube T4 : pipette **40 µL** of Reagent 3 (**vortex vial well before pipetting**).

8.2 Sample dilution

- After blood sample homogenization by tube inversion, add **50 µL** of whole blood in tube T1.
- Homogenize using a vortex for 1 to 2 seconds.

8.3 Immuno-labelling of samples

- In tubes T2 and T3 :
- Pipette **20 µL** of tube T1 content.
- Homogenize the tubes using a Vortex for 1 to 2 seconds.
- Incubate the tubes at room temperature for **10 minutes**.

8.4 Washing

- In each tube T2, T3 and T4 :
- Add **1 mL** of diluted Reagent 1.
- Centrifuge immediately all tubes for **5 minutes** at **1500 g**.
- Discard supernatant.
- Visually check the presence of the cell pellet.
- Add **200 µL** of diluted Reagent 1.
- Resuspend cell pellets by vortex during 1-2 seconds.

8.5 Fluorescent Staining

In each tube T2, T3 and T4 :

- Pipette **20 µL** of Reagent 4.
- Homogenize the tubes using a Vortex for 1 to 2 seconds.
- Incubate all tubes at room temperature for **10 minutes**.
- Add **2 mL** of diluted Reagent 1 in all tubes and store them immediately at 2-8°C until cytometric analysis.

Prepared samples may be stored for maximum **4 hours** at 2-8°C before cytometric analysis.

8.6 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 depending upon the cytometer).

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

Before analysis, homogenize each tube using a Vortex.

• Calibration analysis : tube T4 (Figs 1)

Create a FS LOG vs SS LOG cytogram. Add a discriminator to minimize the background noise.

Set up a gate ("CAL") around the main single bead population (Fig. 1a).

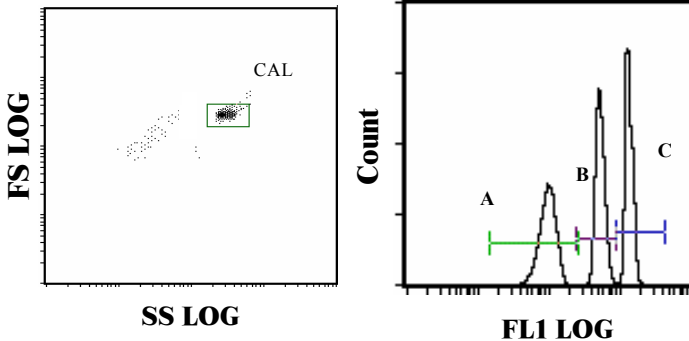
Create a FL1 LOG histogram gated by the "CAL" window.

Note the MFI for each of the 3 fluorescence peaks (Fig. 1b : A, B and C cursors) corresponding to the 3 calibration beads.

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

Fig. 1a : Calibration bead cytogram

Fig. 1b : Cursor settings in the gated fluorescence histogram



• Sample analysis : Tubes T2 and T3 (Figs 2)

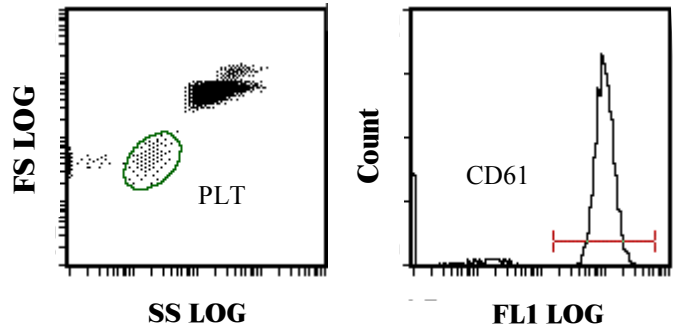
Do not change the acquisition procedure for FL1 (PMT).

On the FS LOG vs SS LOG cytogram (Fig. 2a) platelets are isolated from other whole blood cells by an analysis window "PLT". Check that the discriminator setting does not cut the platelet cloud. **Analyse at least 10,000 events on the window "PLT"**.

In the corresponding gated FL1 fluorescence histogram, note the MFI corresponding to the positive peak of interest for each assay (Fig. 2b). A good analysis allows the obtention of at least 50% positive cells in the window "PLT".

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

Fig. 2b : CD61 immuno-labelling, cursor settings in PLT gated histogram



8.7 Result analysis

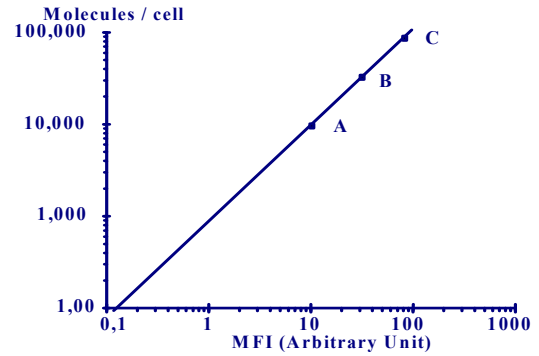
Computer or graphic data analysis.

Plot the LOG₁₀ of the MFI calibration values for the 3 calibration beads (tube T4) on the abscissa (x-axis), and their corresponding LOG₁₀ number of monoclonal antibody molecules (ABC : as indicated on the calibration flyer) on the ordinate (y-axis).

Draw the calibration curve.

Interpolate the LOG₁₀ of the MFI values of tubes T2 and T3 on the calibration curve and read directly their corresponding numbers of monoclonal antibodies.

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



9 RESULT INTERPRETATION

Calculate the HPA-1 ratio, R.

R is the ratio of MAb2 quantitation over MAb1 quantitation (MAb2/MAb1) :

Polymorphism	HPA-1a/1a	HPA-1a/1b	HPA-1b/1b
HPA-1 R	R < 1.20	1.20 ≤ R < 2	R ≥ 2

10 PERFORMANCES

PLT HPA-1 test has been validated on Becton Dickinson instruments type FACSCalibur and Beckman Coulter type XL and XL MCL (System II™ software).

• Test specificity :

The kit PLT HPA-1 has been validated against the Sanger sequencing method and the genotyping method (PCR-SSP) from an evaluation site.

All tested samples (n=181) have been correctly genotyped.

Genotyping distribution of the tested samples (results not published):

Genotype	HPA 1a/1a	HPA 1a/1b	HPA 1b/1b
n	96	57	28
Observed HPA-1 R	0.88 – 1.17	1.21 – 1.52	2.42 – 6.08

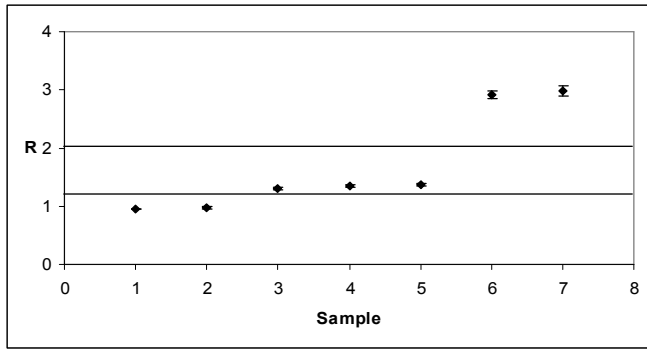
• Reagent specificity :

MAb1 has been clustered as CD61 (Leucocyte typing V) ⁽¹⁾.

MAb2 is specifically directed against GpIIla.

- **Repeatability :**

Samples presenting different genotypes (n = 7) are tested 5 times with the same kit. Variations are represented below (mean +/- 1SD) :

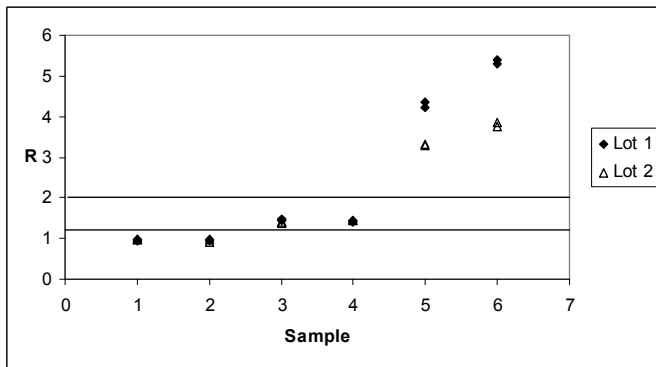


- **Linearity :**

The method linearity is defined for a number of molecules/platelet ranging from the A bead to the C bead values of the calibrator. These values may vary from lot to lot.

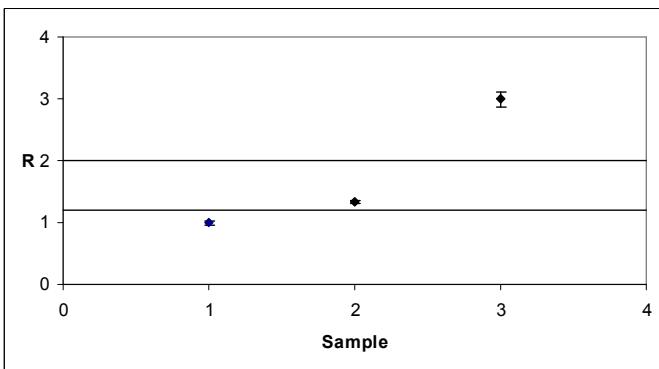
- **Inter lot reproducibility :**

Samples presenting different genotypes (n=6) are tested twice with two 2 different lots. Variations are represented on the following graph :



- **Inter-users reproducibility :**

Samples presenting different genotypes (n=3) are tested with the same kit by 5 different users. Variations are represented on the following graph (mean +/- 1SD) :



11 LIMITATIONS

PLT HPA-1 kit can only be used on samples meeting the following requirements :

- normal GpIIb/IIIa baseline expression obtained with MAb2 (not less than 40,000 GpIIb/IIIa molecules per platelet).
- absence of platelet activation. In order to check the platelet activation state, use the PLATELET Calibrator kit (BioCytex, Ref. 7011) associated with the reagent anti-CD62P (BioCytex, ref. 5111-P).
- absence of GpIIb/IIIa antagonist therapy. Confirm with the PLATELET GpIIb/IIIa Occupancy kit (BioCytex, Ref. 7001), in case of doubt.
- absence of anti GpIIb/IIIa alloantibodies and particularly anti HPA-1a antibodies.
- patient not transfused for 10 days.

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

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4. MIET S. *et al.*, "Evaluation of a new standardized whole blood flow cytometry assay for the determination of the human platelet antigen (HPA)-1." *Thromb Haemost sup.* Aug. 1999, Abstract 2033.
5. CARLSSON LE. *et al.*, "Polymorphisms of the human platelet antigens HPA-1, HPA-2, HPA-3, and HPA-5 on the platelet receptors for fibrinogen (GPIIb/IIIa), von Willebrand factor (GPIb/IX), and collagen (GPIa/IIa) are not correlated with an increased risk for stroke." *Stroke*. 1997, 28:1392-1395.
6. WEISS E.J. *et al.*, "A monoclonal antibody (SZ21) specific for platelet GPIIIa distinguishes P1A1 from P1A2." *Tissue Antigens*. 1995, 46:374-81.
7. METCALFE P. *et al.*, "Nomenclature of human platelet antigens." *Vox Sang*. 2003, 85:240-5.
8. NORTON A. *et al.*, "Review: platelet alloantigens and antibodies and their clinical significance." *Immunohematol*. 2004, 20:89-102.

14 SYMBOLS

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

BIOCYTEX
 140 ch. ARMEE D'AFRIQUE
 13010 MARSEILLE
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