PLT VASP/P2Y12

For the monitoring of specific platelet ADP receptor antagonists

For In Vitro Diagnostic Use

Kit for 10 Tests **Ref. 7014**

コスモ・バイオ株式会社

※本プロトコールは参考用の資料になります。商品ご購入の際は必ず商品に添付されている資料をご参照ください。

研究用

CE ; For US and Canada: For research use only. Not for use in diagnostic procedures.

1 INTRODUCTION

PLT VASP/P2Y12 kit is dedicated to the monitoring of specific platelet ADP receptor (P2Y12) antagonists.

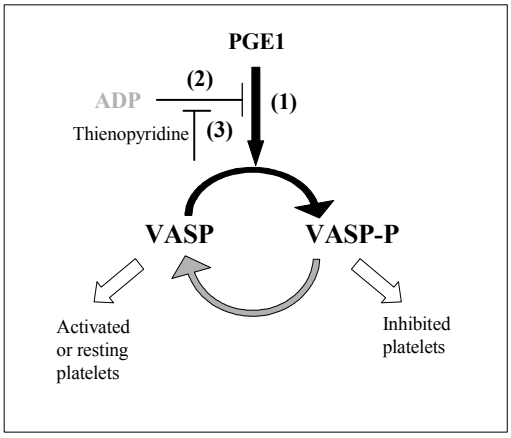
VASP (Vasodilator Stimulated Phosphoprotein) is an intracellular platelet protein which is non phosphorylated at basal state.

VASP phoshorylation is regulated by the cAMP (cyclic Adenosine Monophosphate) cascade. PGE1 (Prostaglandin E1) activates this cascade **(1)** whereas it is inhibited by ADP (Adenosine Diphosphate) through P2Y12 receptors **(2)**.

In the test conditions, VASP phosphorylation correlates with the P2Y12 receptor inhibition, whereas its non-phosphorylation state correlates with the active form of P2Y12 receptor.

Inter-individual variability and resistance to thienopyridine have been widely described [3] [4]. The effect of thienopyridines **(3)** can be demonstrated with **PLT VASP/P2Y12** by the persistence of VASP phosphorylation induced by PGE1 even with the simultaneous addition of ADP.

PLT VASP/P2Y12 could also be used to evaluate *in vitro* effects of P2Y12 receptor antagonists.



2 METHOD

Blood sample is first incubated with PGE1 alone or PGE1 + ADP.

After a cellular permeabilization, VASP under its phosphorylated state is labeled by indirect no wash immunofluorescence using a specific monoclonal antibody (clone 16C2 [6]). Dual color flow cytometry analysis allows to compare the two tested conditions and to evaluate for each sample the capacity of ADP to inhibit VASP phosphorylation.

A platelet reactivity index (PRI) is calculated using corrected mean fluorescence intensities (MFIc) in the presence of PGE1 alone (PGE1) or PGE1 and ADP simultaneously (PGE1+ ADP).

3 KIT REAGENT

- **Reagent 1 :** 1 x 60 mL vial, diluent.
- **Reagent 2a :** 1 vial, PGE1.
- **Reagent 2b :** 1 vial, PGE1 + ADP.
- **Reagent 3 :** 1 x 300 µL vial, fixative.
- **Reagent 4a :** 1 x 200 µL vial, anti VASP-P mouse monoclonal antibody + permeabilization agent.
- **Reagent 4b :** 1 x 100 µL vial, negative isotypic control (mouse monoclonal antibody) + permeabilization agent.
- **Reagent 5 :** 1 x 300 µL vial, staining reagent, polyclonal antibody anti mouse IgG-FITC + platelet counter-staining reagent-PE (anti CD61-PE) + permeabilization agent.

4 MATERIAL REQUIRED BUT NOT PROVIDED

- Stirring machine type vortex.
- Timer.
- Cytometer.
- Adjustable pipettes with disposable tips (10 µL).
- Pipettes (1 or 2 mL).
- Haemolysis tubes for cytometer.
- Distilled water, deionized water or water for injectable solution.

5 REAGENT PREPARATION AND STORAGE

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

Note : Do not freeze the kit.

- **Reagents 1, 3, 4a, 4b and 5 :** ready to use.

Stability after opening : 2 months at 2-8°C when free of contamination.

- **Reagents 2a and 2b :**

Reconstitute each vial with **400 µL** of distilled water and homogenize the tubes using a vortex for 5 seconds.

Stability after reconstitution : 1 month at 2-8°C when free of contamination.

6 WARNING

- Follow the conventional laboratory practices.
- Follow the appropriate reglementation for waste disposal.
- Blood must be considered as potentially infectious.
- Reagent 3 contains between 2% and 5% paraformaldehyde.
  R40 - Limited evidence of a carcinogenic effect.
  R43 - May cause sensitisation by skin contact.
  S36/37 - Wear suitable protective clothing and gloves.
- 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 plastic 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 :**

- Blood sample must be treated within **48 hours** after collection.
- Blood collection tube must be full, stored at room temperature (18-25°C) and unopened before the test.
- The test must be performed on citrated whole blood.

8 PROCEDURE

Note : For good results exercise great care in the **pipeting of small reagent volumes (10 µL) by depositing them at the bottom of the test tubes**.

All reagents must be at room temperature.

We recommend to run a normal sample in parallel of each series as control.

8.1 Reagent tube and sample setup
On a rack, per sample, setup 3 plastic tubes labeled T1, T2 and T3 :
- In tube T1 : pipette **10 µL** of **reagent 2a**.
- In tubes T2 and T3 : pipette **10 µL** of **reagent 2b**.
- In tubes T1, T2 and T3 : pipette **10 µL** of whole blood.
- Homogenize the tubes for 1 to 2 seconds using a vortex **set on low speed**.
- Incubate for **10 minutes** at room temperature.

8.2 Fixation
- In tubes T1, T2 and T3 : pipette **10 µL** of **reagent 3**.
- Homogenize the tubes for 1 to 2 seconds using a vortex **set on low speed**.
- Incubate the tubes at room temperature for **5 minutes**.

8.3 Cell permeabilization and immunolabeling
- In tubes T1 and T2 : pipette **10 µL** of **reagent 4a**.
- In tube T3 : pipette **10 µL** of **reagent 4b**.
- Homogenize the tubes for 1 to 2 seconds using a vortex **set on low speed**.
- Incubate the tubes at room temperature for **5 minutes**.

8.4 Fluorescent staining and platelet counter-staining
- In tubes T1, T2 and T3 : pipette **10 µL** of **reagent 5**.
- Homogenize the tubes using a vortex for 1 to 2 seconds **set on low speed**.
- Incubate the tubes at room temperature for **5 minutes**.
- Add **2 mL** of **reagent 1** in each of the 3 tubes.
- Homogenize the tubes for 1 to 2 seconds using a vortex **set on high speed** and **place them immediately at 2-8°C until the platelet analysis.**

Thus treated, the contents of the tubes may be stored for **2 hours** at **2-8 °C** before platelet 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) statistics is the geometric mean (Mn (y) or GeoMean depending upon the cytometer).
Vortex each tube for 1 to 2 seconds before analysis.
Analyze at least 5,000 platelet events in "B" region.

For the protocol, one FS LOG x SS LOG and one FL1 LOG x FL2 LOG cytograms are necessary.

- Analysis of tube T1 :

- On the FS LOG x SS LOG cytogram isolate the cellular cloud including platelets by the "A" region. Leukocytes (located at the arrow) are excluded from this "A" region (Fig.1).
- The FL1 LOG x FL2 LOG cytogram must be gated by the "A" region.
- Adjust the PMT FL2 in order to get the $FL2^+$ cloud at the beginning of the 3rd decade.
- Adjust the PMT FL1 in order to get the lower part of the $FL1^+$ / $FL2^+$ cloud at the beginning of the 2nd decade.
- **Add a discriminator threshold on FL2 LOG to minimize $FL2^-$ events (background of the instrument and cellular debris).**
- On the FL1 LOG x FL2 LOG cytogram, create a "B" region to isolate the platelets $FL2^+$ from the debris $FL2^-$ (Fig. 2) and note the MFI on the Y-axis.

Note : some samples present a population of debris (comet aspect) located at the left of the cloud of interest (located at the arrow). Set the "B" region during tube T1 analysis in order to include all the platelet cloud and exclude the maximum of debris.

- Analysis of tubes T2 and T3 :

- Do not change the position of the "B" region and the settings of SS, FS, FL1 and FL2 PMT voltages.
- Analyze tubes T2 and T3 and note the MFI on the Y-axis (Figs. 3 and 4).

Figures obtained on Beckman Coulter instrument, type EPICS XL :

Fig. 1 : Setting of the "A" region on tube T1

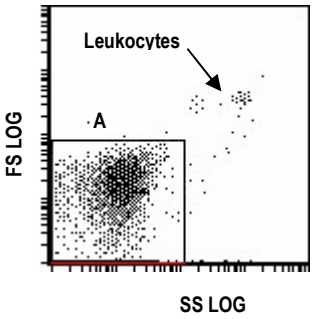


Fig. 2 : Setting of the "B" region on tube T1
(MAb VASP-P, PGE1 condition)

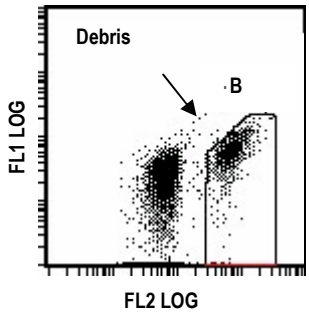


Fig. 3 : Tube T2 analysis
(MAb VASP-P, PGE1+ADP condition)

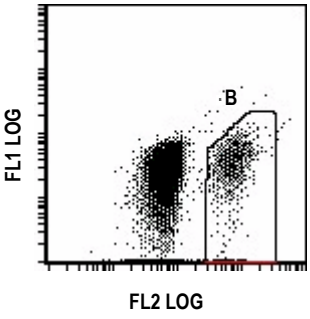
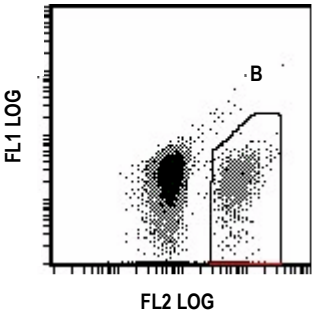


Fig. 4 : Tube T3 analysis
(Negative control, PGE1+ADP condition)



Figures obtained on Becton Dickinson instrument, type FACSCalibur :

Fig. 1 : Setting of the "A" region on tube T1

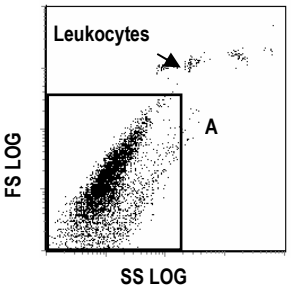


Fig. 2 : Setting of the "B" region on tube T1
(MAb VASP-P, PGE1 condition)

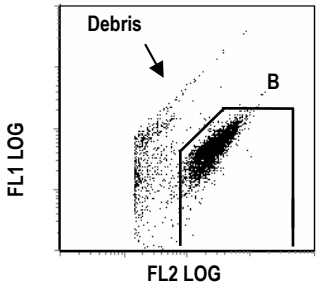


Fig. 3 : Tube T2 analysis
(MAb VASP-P, PGE1+ADP condition)

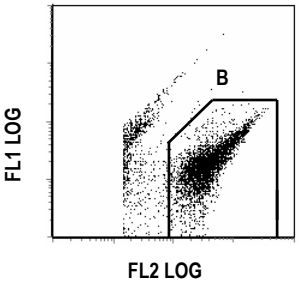
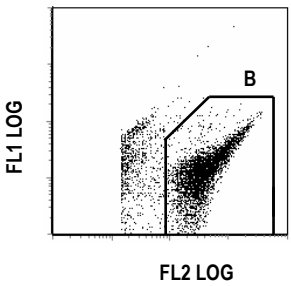


Fig. 4 : Tube T3 analysis
(Negative control, PGE1+ADP condition)



Note : the setting of fluorescence compensation is not required due to the use of the negative isotypic control.
However the setting of fluorescence compensation does not modify the test result.

8.6 Analysis of the results

After cytometric analysis, determine the value of the "corrected" MFI (MFI_C) for tubes T1 and T2.

MFI_C is obtained after subtraction of the MFI value obtained for the negative control (tube T3) to the MFI value obtained for MAb anti VASP-P (tubes T1 or T2).

$MFIc_{(PGE1)} = MFIc_{(T1)} = MFI\ (T1) – MFI\ (T3)$
$MFIc_{(PGE1 + ADP)} = MFIc_{(T2)} = MFI\ (T2) – MFI\ (T3)$

9 RESULT INTERPRETATION

A platelet reactivity index (PRI) is calculated using corrected mean fluorescence intensities (MFIc) in the presence of PGE1 alone $_{(PGE1)}$ or PGE1 and ADP simultaneously $_{(PGE1+ ADP)}$ according to the following calculation :

Platelet reactivity index (PRI) =
$[(MFIc_{PGE1} – MFIc_{(PGE1 + ADP)}) / MFIc_{PGE1}] \times 100$

Each laboratory must establish its own interpretation values specific from the P2Y12 antagonist to evaluate.

Treatment with the clopidogrel :

Inter-individual variability in the response to clopidogrel has been demonstrated using **PLT VASP/P2Y12** kit [1].

The PRI of the patients with ischemic cardiovascular disease (n=33) treated with clopidogrel for more than 1 week, vary from 6.6% to 85.8%.

Bad responders to clopidogrel **85.8%** → **6.6 %** Good responders to clopidogrel

In order to measure the efficiency of a P2Y12 antagonist such as clopidogrel, apply the following recommendation :

1- Determine the basal PRI range (expressed as mean +/- 2 SD (standard deviation)) on a group of patients relevant of the disease of interest and not receiving the P2Y12 antagonist to evaluate.
As a guide, according to the publication of Aleil B. *et al.* [1], the PRI of the patients (n=34) with ischemic cardiovascular disease, not receiving clopidogrel is 79.0 +/- 4.1% (expressed as mean +/- SD).

2- Determine the basal PRI value of the patient to be tested before the treatment (PRI_0) and confirm that this value is included in the basal PRI range pre-established. Otherwise, refer to the paragraph limitations (§11) and repeat the test if necessary.

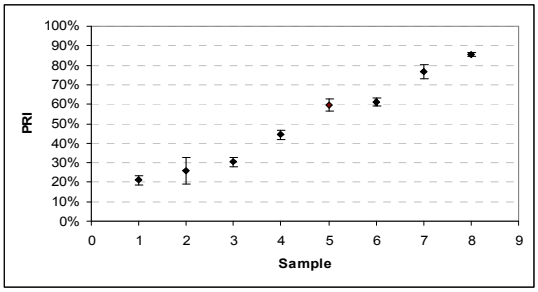
3- Determine the PRI value at a time point T (PRI_T) according to the pharmacodynamic properties of the P2Y12 antagonist evaluated. A PRI_T value which is still included in the basal PRI range signifies that the patient has not responded to the drug.

10 PERFORMANCES

PLT VASP/P2Y12 has been validated on Becton Dickinson instrument type FACSCalibur and Beckman Coulter instrument type XL and XL MCL.

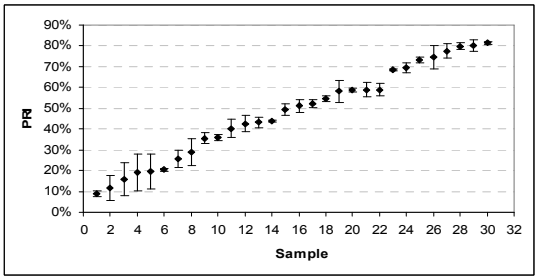
- **Repeatability :**

Samples (n = 8) presenting different level of VASP response (PRI) are tested 5 times with the same kit. Variations are represented below (mean +/- SD) :



- **Inter lot reproducibility :**

Samples (n = 30) presenting different level of VASP response (PRI) are tested with 1 kit from 3 different lots. Variations are represented on the following graph (mean +/- SD) :



- **Working range :**

The working range for this method is from 0 up to 100 % of PRI.

- **Interferences :**

- Aspirin :

According to the publication from Aleil B. *et al* [1], Aspirin has no significant interference on **PLT VASP/P2Y12** assay (n=67) : p=0.328.

- Platelet count :

On non treated samples from 50,000 up to 300,000 platelets/µL blood the platelet count has no significant interference on **PLT VASP/P2Y12** assay.

- Abciximab :

According to the publication from Van Werkum J. *et al* [2], abciximab has no significant interference on **PLT VASP/P2Y12** assay (n=11) : p=0.89.

- **Aggregation correlation :**

According to the publication from Aleil B. *et al* [1], **PLT VASP/P2Y12** test is strongly correlated with the inhibition of ADP-induced platelet aggregation due to in vitro specific P2Y12 blockade : r = 0.72 ; p < 0.0001.

11 LIMITATIONS

- **PLT VASP/P2Y12** kit cannot be used on hemolyzed blood samples.
- **PLT VASP/P2Y12** kit should not be used on samples presenting a red blood cell count lower than the normal values. In this case it is recommended to repeat **PLT VASP/P2Y12** assay on a new sample collected at least 24 hours later.
- The immediate platelet analysis right after the final addition of the 2 mL of reagent 1 may reveal an incomplete red cell lysis for some samples. An overlap between the platelet cloud and the red blood cell cloud then occurs.
In order to get a complete red cell lysis store the sample for 5 minutes at room temperature before vortexing the tubes and performing the cytometric analysis again.

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

(1) ALEIL B. *et al.* (2005) *J Thromb Haemost* 3 : 85-92.
(2) VAN WERKUM J. *et al*. (2007) *J Thromb Haemost* 5 : 881-883.
(3) BONELLO L. *et al*. (2009) *Am J Cardiol*. 103(1):5-10.
(4) BARRAGAN P. *et al.* (2003) *Cathet Cardiovasc Intervent* 59 : 295-302.
(5) GURBEL P.A. *et al.* (2003) *Circulation* 107 : 2908-2913.
(6) MULLER I. *et al.* (2003) *Thromb Haemost* 89 : 783-787.
(7) GEIGER J. *et al.* (1999) *Arterioscler Thromb Vasc Biol* 19 : 2007-2011.
(8) SCHWARZ U.R. *et al.* (1999) *Thromb Haemost* 82 : 1145-1152.

14 NOTICE TO PURCHASER

PLT VASP/P2Y12 kit is covered by patent WO 99/24473.

15 SYMBOLS

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

BIOCYTEX
140 ch. DE L'ARMEE D'AFRIQUE
13010 MARSEILLE
FRANCE
TEL : +33 (0) 4 96 12 20 40
FAX : +33 (0) 4 91 47 24 71

Version May 2010