CY-QUANT VASP/P2Y12

For the measurement of specific
platelet ADP receptor antagonists

Kit contents:

- 1 microtiter plate of Reagent 1 (96 Anti-VASP coated wells)
- 3 vials of Reagent 2a (PGE1)
- 3 vials of Reagent 2b (PGE1 + ADP)
- 1 vial of Reagent 3 (Lysis buffer)
- 1 vial of Reagent 4 (Washing solution)
- 1 vial of Reagent 5 (Dilution buffer)
- 1 vial of Reagent 6 (Anti-VASP-P peroxidase)
- 1 vial of Reagent 7 (TMB)
- 1 vial of Reagent 8 (Stop solution)
- 1 tool to extract wells from their strips

For In Vitro Diagnostic Use

Ref. 7502

CE

1- INTRODUCTION

CY-QUANT VASP/P2Y12 is an enzyme-linked immunosorbent assay (ELISA) procedure for the determination of serine 239-phosphorylated VASP (VASP-P) in platelets from fresh human whole blood.

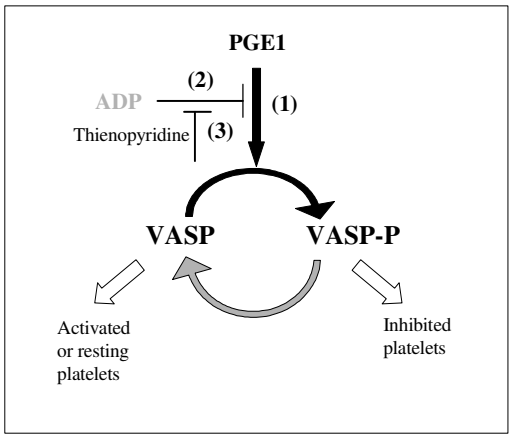
CY-QUANT VASP/P2Y12 kit is dedicated to the measurement of specific platelet ADP receptor (P2Y12) antagonists.

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

Prostaglandin E1 (PGE1) induces phosphorylation of VASP **(1)** whereas the binding of adenosine diphosphate (ADP) to P2Y12 receptors **(2)** leads to dephosphorylation of VASP. Under test conditions, in the concomitant addition of ADP and PGE1, the effect of ADP dominates and leads to VASP dephosphorylation, unless the P2Y12 receptor is efficiently blocked by antiplatelet drugs targeting this receptor (such as thienopyridines). Thus, the level of VASP phosphorylation in this condition reflects the level of P2Y12 receptor inhibition.

Inter-individual variability and resistance to antiplatelet drugs have been widely described [a,b]. The effect of thienopyridines **(3)** can be demonstrated with **CY-QUANT VASP/P2Y12** by the persistence of VASP phosphorylation induced by PGE1 despite simultaneous addition of ADP.

CY-QUANT VASP/P2Y12 may also be used to evaluate *in vitro* effects of P2Y12 receptor antagonists.



2- TEST PRINCIPLE

After a first step of parallel whole blood sample activation with PGE1 and PGE1+ADP (Reagent 2a and 2b), platelets from the sample are lysed (Reagent 3), allowing released VASP to be captured by an anti-human VASP antibody which is coated in the microtiter plate (Reagent 1). Then, a peroxidase-coupled anti-human VASP-P antibody (Reagent 6) binds to phosphorylated serine 239 antigenic determinant of VASP. The bound enzyme peroxidase is then revealed by its activity on TMB substrate (Reagent 7) over a predetermined time. After stopping the reaction (Reagent 8), absorbance at 450 nm is directly related to the concentration of VASP-P contained in the sample.

A platelet reactivity index (PRI) is calculated using optical density (OD_{450nm}) in the presence of PGE1 alone [*PGE*1] or PGE1 and ADP simultaneously [*PGE*1+ *ADP*].

3- REAGENTS

- **Reagent 1:** 96-wells microtiter plate composed of 12 breakable strips of 8 wells coated with mouse anti-human VASP MAb, in a re-sealable pouch.
- **Reagent 2a:** vial, lyophilized PGE1.
- **Reagent 2b:** vial, lyophilized PGE1 + ADP.
- **Reagent 3:** vial, 15 mL, lysis buffer.
- **Reagent 4:** vial, 50 mL, 20 fold-concentrated washing solution.
- **Reagent 5:** vial, 50 mL, dilution buffer.
- **Reagent 6:** vial, 1.6 mL, 20 fold-concentrated specific mouse anti-human VASP-P MAb coupled with peroxidase.
- **Reagent 7:** vial, 25 mL, TMB (tetramethylbenzidine).
- **Reagent 8:** vial, 15 mL, stop solution.

4- MATERIAL REQUIRED BUT NOT PROVIDED

- Deionized or distilled water, equilibrated at room temperature and preferably sterile.
- Timer.
- Multi-channel pipettes, pipettes with disposable tips.
- ELISA plate reader set at 450 nm.
- Vortex.
- Absorbent paper.

5- WARNING

- Follow the conventional laboratory practices.
- Follow the appropriate regulation for waste disposal.
- Blood must be considered as potentially infectious.
- Reagent 3 – Lysis buffer:
  H319: Causes serious eye irritation
  H333: May be harmful if inhaled
  EUH208: Contains 5-chloro-2-methyl-4-isothiazol-3-one/2-methyl-4-isothiazol-3-one (3:1). May produce an allergic reaction
  P280: Wear protective gloves/protective clothing/eye protection/face protection
  P305 + P351 + P338: IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing
  P304 + P340: IF INHALED: Remove person to fresh air and keep comfortable for breathing
- Reagent 5 – Dilution buffer:
  H317: May cause an allergic skin reaction
  P280: Wear protective gloves / protective clothing / eye protection / face protection
  P302 + P352: IF ON SKIN: Wash with plenty of soap and water

- Reagent 8 – Stop solution:
  H314: Causes severe skin burns and eye damage
  P280: Wear protective gloves/protective clothing/eye protection/face protection
  P301 + P330 + P331: IF SWALLOWED: rinse mouth. Do NOT induce vomiting
  P303 + P361 + P353: IF ON SKIN (or hair): Remove/Take off immediately all contaminated clothing. Rinse skin with water/shower
  P305 + P351 + P338: IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing
  P310: Immediately call a POISON CENTER or doctor/physician

6- REAGENTS PREPARATION AND STORAGE

- When stored at 2-8° C, unopened kits and contents remain stable until the printed expiration date.
- Before use, all reagents must be equilibrated at room temperature (RT, 18-25 °C) for at least 30 minutes.

● Reagent 1
Ready-to-use. After first use, immediately replace unused strips and wells in the re-sealable pouch with the desiccant and store at 2-8°C.
Stability after opening: 2 months at 2-8°C, when free of contamination.

● Reagents 2a and 2b
Reconstitute each vial with **900 µL** of deionized or distilled water and homogenize the content using a vortex for 5 seconds.
Stability after reconstitution: 1 month at 2-8°C, when free of contamination.

● Reagents 3, 5 and 8
Ready-to-use. Stability after opening: 2 months at 2-8°C, when free of contamination.

● Reagent 4
Stability after opening: 2 months at 2-8°C, when free of contamination.
Before use, dilute the reagent **1:20** with deionized or distilled water.
For one well, dilute 100 µL of **Reagent 4** with 1 900 µL of deionized or distilled water.
Stability after dilution: 15 days at 2-8°C, when free of contamination.
Note: The presence of crystals does not affect the quality of the reagent. If necessary, warm at 37°C until all crystals have dissolved. Then, homogenize and equilibrate at RT.

● Reagent 6
Stability after opening: 2 months at 2-8°C, when free of contamination.
Before use, dilute the reagent **1:20** with **Reagent 5**.
For one well, dilute 15 µL of **Reagent 6** with 285 µL of **Reagent 5**.
Stability after dilution: 1 hour at RT.
Note: Since the vial is filled to capacity, pipette carefully to avoid reagent overflow.

● Reagent 7
Ready-to-use. Stability after opening: 2 months at 2-8°C, when free of contamination.
Note: Avoid exposure to light, heat and contamination with metal ions or peroxidase.

7- SPECIMEN COLLECTION AND STORAGE

- Draw venous whole blood into a **0.109 M trisodium citrate** collection tube, according to manufacturer's instructions.
- Maintain platelet integrity. Avoid platelet activation during the collection procedure (shaking, heat shock).
- Blood collection tube must be filled to capacity, stored at RT and unopened before the test.
- Samples must be analyzed within **24 hours** from collection.

8- PROCEDURE

We recommend to test a normal sample in parallel of each series, to serve as a control.

Notes:
- The washing steps can be performed either with an automated plate-washing equipment or manually with a multi-channel pipette.
- During manual washing, first empty all wells by flicking off the liquid into an appropriate container and blot the plate on a clean absorbent paper. Then, fill each well with 300 µL of diluted Reagent 4, flick off the washing solution, and blot the plate on a clean adsorbent paper.
- The number of washing steps must be scrupulously respected.
- Do not leave the wells dry at any time.
- Do not expose the strips to strong light.
- Check the absence of bubbles in the wells before OD measurement.

8.1- OPERATING PROCEDURE

At each step, identical incubation time must be carefully respected for each well.
Distribute the samples to be tested and the blank in duplicate; one duplicate blank is enough for a series of samples.
Pipette directly into precoated wells (Reagent 1):

		Well **PGE1**	Well **PGE1 + ADP**	Well **Blank**
ANTIGEN CAPTURE	Reagent:	**2a**: 40 µL	**2b**: 40 µL	**5**: 180 µL
	Whole blood sample:	40 µL	40 µL	—
	Thoroughly mix the contents of each well by pipetting up and down 8-10 times			—
	Cover the wells and incubate **10 minutes** at RT			—
	Reagent:	**3**: 100 µL	**3**: 100 µL	—
	Thoroughly mix the contents of each well by pipetting up and down 8-10 times			
	Cover the wells and incubate **30 minutes** at RT			
Wash all wells 3 times with 300 µL of diluted Reagent **4**, then add immediately:				
CONJUGATE IMMOBILIZATION	Diluted Reagent **6**:	200 µL	200 µL	200 µL
	Cover the wells and incubate **30 minutes** at RT			
Wash all wells 3 times with 300 µL of diluted Reagent **4**, then add immediately:				
COLOR DEVELOPMENT	Reagent **7**:	200 µL	200 µL	200 µL
	Incubate **5 minutes** at RT, and then add:			
	Reagent **8**:	100 µL	100 µL	100 µL
	Thoroughly homogenize the contents of each well			
OD PLATE MEASUREMENT	Measure the absorbance at **450 nm** up to **4 hours** at RT after stopping the reaction			

8.2- PLATELET REACTIVITY INDEX (PRI) CALCULATION

A platelet reactivity index (PRI) is calculated using optical density (OD_{450nm}) in the presence of PGE1 alone [*PGE*1] or PGE1 and ADP simultaneously [*PGE*1+ *ADP*], according to the following formula:

$$PRI\ (\%) = \frac{OD_{450nm}[PGE1] - OD_{450nm}[PGE1 + ADP]}{OD_{450nm}[PGE1] - OD_{450nm}[Blank]} \times 100$$

Note: Each laboratory must establish its own interpretation values depending upon the P2Y12 antagonist to evaluate.

In order to measure the efficiency of a P2Y12 antagonist, apply the following recommendations:

1- Determine the basal PRI range (mean ± 2 standard deviations) on a group of patients relevant of the disease of interest and not receiving the P2Y12 antagonist to evaluate. As a guide, the PRI of untreated healthy donors (n=32) is ranging from 89% to 99% (data from external study).

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 pre-established basal PRI range. Otherwise, refer to the Limitations paragraph (§10) 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.

In summary, a low PRI corresponds to a good responder patient while a high PRI stands either for a healthy subject or a bad responder patient. The lower the PRI, the higher P2Y12 receptor inhibition.

9- PERFORMANCES

Repeatability:

Two samples presenting different levels of PRI are tested 8 times with the same kit:

Sample	Sample 1	Sample 2
n	8	8
$\bar{X}$ (PRI %)	43,69%	97,85%
SD	2,02	0,57
CV	4,6%	0,6%

Working range:

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

Correlation to PLT VASP/P2Y12 (BioCytex ref. 7014, CE):

CY-QUANT VASP/P2Y12 test is strongly correlated with the flow cytometric PLT VASP/P2Y12 test: n = 96; r = 0.95; p < 0.001.

Interferences:

- Platelet count: on samples from 50,000 up to 375,000 platelets/µL, the platelet count has no significant interference on CY-QUANT VASP/P2Y12 assay.

- Red blood cell count: on non treated samples from 1 x 10^6 up to 5,8 x 10^6 red blood cells/µL, the red blood cell count has no significant interference on CY-QUANT VASP/P2Y12 assay.

- Aspirin and anti GpIIb/IIIa drugs have no significant interference on CY-QUANT VASP/P2Y12 assay since VASP biomarker is specific from the P2Y12 signaling pathway.

10- LIMITATIONS

CY-QUANT VASP/P2Y12 kit cannot be used on activated and/or hemolyzed blood samples.

11- LIABILITY

The *in vitro* diagnostic use is only valid within the strict application of the package insert. Any modification of the protocol may influence the result of the test.

Never switch or mix vials originating from different kits.

In cases where these recommendations are not strictly respected, no contestation or replacement of the product will be accepted.

12- REFERENCES

(a) Gurbel PA. *et al*. (2007) *Thromb Research* 120:311-321.
(b) Angiollilo D. *et al*. (2007) *J Am Coll Cardiol* 49:1505-1516.
(c) Barragan P. *et al*. (2010) *Thromb. Haemost* 104(2): 410-11.
(d) Jakubowski J.A. *et al*. (2012) *Thromb. Haemost* 107: 388-395.
(e) Abtan J. *et al*. (2013) *Thromb Haemost* 110(5):1055-64.

13- SYMBOLS

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

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