

Customer Information Sheet

VASPFix

 (catalogue no. PSR-003)

A single-step solution to monitor phosphorylation of vasodilator-stimulated phosphoprotein (VASP)

Use VASPFix undiluted at a ratio of 5 volumes of VASPFix to 1 volume of sample. This 5:1 ratio has been tested with human whole blood and platelet-rich plasma (PRP). VASPFix may be suitable for monitoring VASP phosphorylation in other cell types, and for use with species other than humans.

INTENDED USE

VASPFix was developed to monitor phosphorylation of vasodilator-stimulated phosphoprotein (VASP) in human platelets using a single solution that could be added to blood or PRP without any need for multiple steps in the analytical process. In addition, the treated sample can be stored for analysis at a later stage should this be required. The level of phosphorylated VASP (VASP-P) in platelets is then determined using flow cytometric analysis.

SUMMARY AND EXPLANATION

The level of VASP-P in platelets is regulated by agents that both stimulate and inhibit adenylate and guanylate cyclase, which produce cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), respectively. In turn, cAMP and cGMP bring about phosphorylation of VASP. Measurement of VASP-P can be used to monitor the effects of agents that act at cell-surface receptors linked to stimulation or inhibition of adenylate cyclase and agents that regulate the activity of phosphodiesterases.

VASPFix contains all that is needed to monitor changes in VASP-P in platelets at the point of addition to blood or PRP. The cells are lysed, the VASP and VASP-P is released and adsorbed to coated beads, a fluorescently labelled antibody binds to the VASP-P (at Ser157) on the coated beads, and the amount of VASP-P is then determined by flow cytometric analysis. If necessary, the treated samples can be stored frozen (-20°C) for analysis at least up to 6 months after sample preparation.

PRINCIPLES OF THE PROCEDURE

The principles of the procedure are outlined below:



At the point in an experiment at which the level of VASP-P in platelets is to be determined, VASPFix is added to the blood or PRP. The sample is then incubated in the dark for 2 hours after which the fluorescence intensity of the beads in the sample is determined by flow cytometry.

REAGENT

The VASPFix reagent is provided in 1ml bottles. VASPFix should be used undiluted in a 5:1 ratio with the suspension of cells that are to be analysed.

Typically 5µl of the treated sample – whole blood or PRP – is added to 25µl of VASPFix, but larger volumes can also be used.

PRECAUTIONS

1. VASPFix is provided for *in vitro* use only.
2. **WARNING:** VASPFix contains a number of different chemicals that could potentially have adverse effects in humans. Keep VASPFix out of the reach of children. Keep the container in a well-ventilated place. Wear suitable protective clothing and gloves. If swallowed, seek medical advice immediately and show this information. Dispose of VASPFix according to local regulations for hazardous solutions.
3. All patient specimens and materials with which they come into contact are considered biohazards and should be handled as if capable of transmitting infection.

STORAGE AND HANDLING

On receipt VASPFix should be stored at -20°C until needed and following thawing it should be stored at 4°C and used within 1 month. It should be protected from light. VASPFix should be thoroughly vortexed before each use to ensure an equal distribution of all components. Stability studies are continuing but VASPFix is stable at -20°C for at least 6 months. Do not use the VASPFix if the bottle becomes contaminated or precipitation occurs.

SUGGESTED PROCEDURES

Sample preparation

Anticoagulated blood may be treated with the agent(s) under investigation either at room temperature or 37°C. Following mixing of sample with the required agents it should be left for the desired time (suggested time 2-6 minutes) and then the reaction is stopped by mixing the sample with VASPFix.

Sample treatment can be performed in any suitable tube. VASPFix should always be used at a ratio of 5:1. After addition of VASPFix the samples should be vortexed (to ensure cell lysis) and then incubated in the dark at room temperature for 2 hours prior to analysis by flow cytometry. Alternatively they can be capped or sealed and immediately frozen for analysis at a later time point (recommended within 6 months). In the latter case, the samples should be removed from the freezer and incubated in the dark at room temperature for 2 hours prior to flow cytometric analysis.

Flow cytometric analysis

The VASP-P is quantitated on coated beads with VASP and VASP-P adsorbed on their surface and with anti-VASP-P antibody bound to the phosphorylated form of the protein (conjugated to a FITC fluorochrome). The beads themselves have APC fluorescence and can be easily identified from the debris, therefore it is preferable to use a cytometer with blue and red lasers. However, beads can also be identified on the basis of their forward and side scatter characteristics. In each sample 300 beads are analysed, gated as shown in Figure 1. until staining and analysis. If they need to be transported, samples may be placed in a package, appropriately labelled and posted. Samples should be analysed between 24 hours and up to 9 days following fixation.

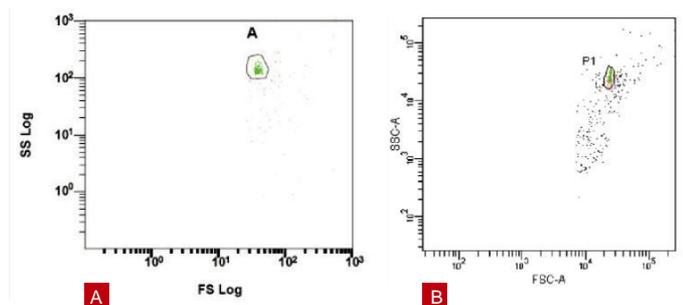


Figure 1. Typical forward scatter/side scatter dot plots obtained on (A) Beckman Coulter instrument (FC500) and (B) Becton Dickinson instrument (LSR II). Beads are shown in regions A or P1.

PMT voltages should be set for APC so that the beads appear in the beginning of the third decade (middle of the axis) and for FITC so that beads from samples with low VASP-P levels appear in the second decade (Figure 2). Discriminator threshold could be set to minimize debris in the analysis, but this should be done with caution so as not to exclude any beads from the analysis.

A rectangular gate should be drawn around the bead population on the APC Log (FL4 Log) x FITC Log (FL1 Log) dot plot as shown in Figure 2.

Prior to the analysis of each tube add diluent buffer (the amount will depend on the type of cytometer, in our experience it varies from 40µl to 400µl). Vortex thoroughly for 3-5 seconds just prior to flow cytometric analysis. Record the FITC Median Fluorescence (MF) of the acquired 300 beads.

For more details and assistance please contact Platelet Solutions Ltd.

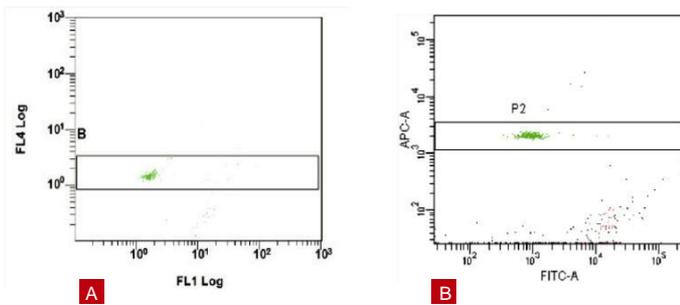


Figure 2. Typical APC Log (FL4 Log) x FITC Log (FL1 Log) dot plots obtained on (A) Beckman Coulter instrument (FC500) and (B) Becton Dickinson instrument (LSR II).

The height of this gate should be just sufficient to include only the beads and the width should be wide enough to allow for low and high FITC fluorescence level (i.e. low and high VASP-P levels). With an increase in VASP-P the beads will shift right along the FITC axis as shown in Figure 3.

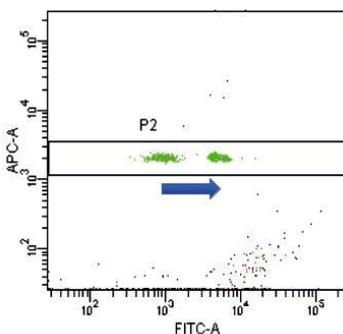


Figure 3. Overlay of graphs obtained with samples containing low and high VASP-P levels. An increase in VASP-P level results in an increase in bead-associated FITC fluorescence and a shift to the right.

LIMITATIONS

Laboratories should establish their own normal reference ranges for their procedures, agonists, patient populations and flow cytometers. The absolute MF values may vary between different batches of VASPFix so we recommend including an internal control with maximum VASP-P level and expressing all data as percentage of the maximum or expressing data as a percentage change between different samples/conditions.

The stability of VASPFix-treated samples has been assessed extensively using human whole blood and PRP with the procedures described here. At this stage studies on cells and tissues other than platelets or using blood from other species have not been performed.

PERFORMANCE CHARACTERISTICS

Studies have been performed using blood and PRP from healthy volunteers, patients with acute coronary syndromes and patients with stroke; some of these were treated with a P2Y12 antagonist. The level of VASP-P has been used to determine the effects of agents that stimulate adenylate cyclase (e.g. prostacyclin, the prostacyclin mimetic iloprost and PGE1 [acting via the IP receptor], PGE2 [acting via the EP4 receptor], and adenosine [acting via the A2A receptor]). VASP phosphorylation has also been used to demonstrate the effects of agents that inhibit adenylate cyclase (e.g. ADP [acting via the P2Y12 receptor], and PGE1 and PGE2 [acting via the EP3 receptor]).

Studies in whole blood and PRP were found to provide different absolute values of VASP-P fluorescence in the presence of (e.g.) iloprost, but the results were identical when they were expressed as % of maximum VASP-P in response to the agent. Studies in cardiovascular patients receiving a P2Y12 antagonist such as clopidogrel or prasugrel provided a platelet reactivity index (PRI) that reflected the potency and variability of the drug being administered. For more details see the paper by Glenn et al 2014 (below).

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