Customer Information Sheet

PAMFix (catalogue no. PSR-001)

For fixation of Platelet Activation Markers (PAM) such as P-selectin (CD62P) prior to immunofluorescence staining and flow cytometric analysis.

Use PAMFix undiluted at a ratio of 2 volumes of PAMFix to 1 volume of sample requiring fixation. This 2:1 ratio has been tested with human whole blood and platelet-rich plasma (PRP). PAMFix may be suitable for fixation of other biomarkers on platelets or other cell types, and for use with species other than humans.

INTENDED USE

PAMFix was developed for measurement of P-selectin (CD62P), a platelet activation marker (PAM). The intention was to fix P-selectin on activated platelets so that samples can be analysed between 24 hours and 9 days following fixation. This enables blood samples to be processed and fixed remotely and posted to a central laboratory where P-selectin is quantified using immunofluorescence staining and flow cytometric analysis.

SUMMARY AND EXPLANATION

When platelets are activated P-selectin, which is normally present in α-granules, appears on the platelet surface and the amount present provides a measure of the degree of platelet activation. However, P-selectin expression on the platelet surface can be transient and, if unfixed, P-selectin can be cleaved or re-internalised. Extensive testing has shown that PAMFix effectively fixes the P-selectin on the platelet surface. PAMFix was developed to provide stable readings when the P-selectin expression is analysed between 24 hours and 9 days following fixation.

PRINCIPLES OF THE PROCEDURE

When PAMFix is added to whole blood or PRP the ratio of the PAMFix to sample should be 2:1. The fixed samples may then be transported and/or stored at room temperature or in a fridge prior to flow cytometric analysis.

Just prior to analysis the samples are stained with a fluorescently-labelled antibody against P-selectin. PAMFix has been tested extensively where P-selectin expression on human platelets is measured using CD62P-FITC from Serotec (cat. no. MCA796F). An additional antibody labelled with a different fluorochrome can also be used against another platelet-specific protein when it is deemed necessary to identify the platelets separately from other blood cells; CD61-PerCP from BD (cat. no. 347408) or CD61-PE from Miltenyi Biotec (cat. no. 130-081-501) are examples of such platelet identifiers. P-selectin expression can be quantified either as % of platelets that are positive for P-selectin or as the median fluorescence of the entire platelet population.

PAMFix has also been used for measurement of CD63 expression as an alternative to P-selectin as an activation marker. CD63 is a measure of secretion from storage granules known as dense bodies in platelets, whereas P-selectin emerges from α-granules. Specific information on which antibodies to use can be provided on request.

There is some experience of measurements of P-selectin on platelets from species other than humans for which use of different antibodies may be preferable. Specific information can be provided on request.

REAGENT

The PAMFix reagent is provided in 100ml bottles. PAMFix should be used undiluted in a 2:1 ratio with the suspension of cells that are to be fixed. Typically 1ml is added to 0.5ml of whole blood or PRP following platelet stimulation with an agonist, but smaller or larger volumes can also be used.

PRECAUTIONS

1. PAMFix is provided for *in vitro* use only
2. WARNING: PAMFix contains a small quantity of formaldehyde. Formaldehyde is allergenic and exposure can cause cancer. Formaldehyde is harmful by inhalation, in contact with skin and if swallowed. It is irritating to eyes and skin. There is a possible risk of irreversible effects.
   Formaldehyde may cause sensitisation by skin contact. Consequently keep PAMFix 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 PAMFix 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

PAMFix is stable for at least 18 months at 4ºC or room temperature, USE BEFORE EXPIRY DATE. Do not use the PAMFix if the bottle becomes contaminated or precipitation occurs.

SUGGESTED PROCEDURES

Blood Collection
Blood is collected into a suitable anticoagulant; trisodium citrate dihydrate (3.13% w/v, 1ml per 9ml blood) is often used although other suitable anticoagulants such as acid-citrate-dextrose, P-PACK, hirudin or heparin may be used. Tubes are mixed carefully to ensure adequate dispersal of the anticoagulant. Blood may be left at room temperature for a fixed time period (e.g. 10-60 minutes) or processed immediately after venepuncture. Blood may be collected directly into PAMFix to measure basal levels of P-selectin immediately after venepuncture; this can be done without prior use of an anticoagulant.

Platelet Activation
Anticoagulated blood may be stimulated with platelet agonist(s) either at room temperature or 37°C. All reagents, samples and tubes should be pre-warmed for at least 10 minutes prior to use if experiments are performed at 37°C. Following mixing of sample with the agonist it should be left undisturbed for the desired time (suggested time 2-6 minutes) and then fixed by addition of PAMFix. Since P-selectin is measured on the surface of single platelets it is advised to perform platelet stimulation in whole blood or PRP in the presence of EDTA (final concentration 4mM) to avoid the formation of platelet aggregates. Dilution of whole blood or PRP by at least 50% (for example with saline) can also help to prevent the formation of platelet aggregates when EDTA has not been used.

Platelet activation can be carried out in any suitable tube or even a 96 well plate. PAMFix should always be used at a ratio of 2:1 with the sample undergoing fixation. All efforts should be taken to prevent the formation of platelet aggregates by using EDTA or dilution of the sample as described above. After fixation the samples should be mixed well and capped or sealed.

Fixed samples may be stored at room temperature or 4ºC 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.

LIMITATIONS

Laboratories should establish their own normal reference ranges for their procedures, agonists, patient populations and flow cytometers. Sample stability has been assessed extensively for measurements of P-selectin using human whole blood and PRP with the procedures described here. Studies performed on CD63 expression and using blood from other species has not been fully investigated.

PERFORMANCE CHARACTERISTICS

Stability studies have been performed using blood from healthy volunteers, patients with acute coronary syndromes and patients with stroke; some of these were treated with aspirin and/or a P2Y12 antagonist. The following agonists were used for these stability studies: arachidonic acid in combination with epinephrine, and adenosine diphosphate in combination with the thromboxane A2 analogue U46619. EDTA was added to the blood along with the agonists to prevent platelet aggregate formation.

Stability studies have shown that samples may be stored in a fridge or at room temperature for between 24 hours and 9 days. Spearman correlations for P-selectin expression measured as % platelets that are positive for P-selectin or median fluorescence for samples analysed 24 hours after fixation and after 9 days following storage at room temperature gave R=0.899 and R=0.953, respectively ($p<0.0001$). A similar comparison of samples stored at 4^{o}C gave R=0.962 and R=0.984 ($p<0.0001$).

Transport studies have shown that samples can be posted through the Royal Mail system. Spearman correlations for P-selectin expression measured as % platelets that are positive for P-selectin or median fluorescence for samples analysed 24 hours after fixation and again up to 7 days in the postal system gave R=0.932 and R=0.955, respectively ($p<0.0001$). The effects of severe adverse weather conditions that may cause freezing or result in samples being subjected to higher than average temperatures for extended periods of time have not been fully tested.

TECHNICAL SUPPORT

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