Standard Operating Procedure (SOP)

Short Title: **Rapid γH2AX processing**

This SOP replaced the version of . . : –

Notes of change:

Aim: Define standard procedures and conditions for rapidly obtaining gamma-H2AX-immunostained blood samples suitable for high throughput biological dosimetry based on microscopic foci scoring.

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substitutes: –

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Attachments: none

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1. Aim of the method:	Define standard procedures and conditions for rapidly obtaining gamma-H2AX-immunostained blood samples suitable for high throughput biological dosimetry based on microscopic foci scoring.
1.1. Clinical Indications: (mention in cases)	>20 individuals with suspected exposure to a significant fraction (>10%) of lymphocytes in the body to >0.5 Gy ionising radiation less than four days ago
2. Background:	Immunofluorescence staining of DNA double-strand break-associated nuclear foci enables rapid detection of recent radiation exposure in blood cells
3. Sample requirements:	
3.1 Nature of sample	Blood, typically obtained by venipuncture
3.2 Container:	Blood collection tubes with heparin or EDTA anti-coagulant
3.3 Minimum volume	90 µl
3.4 Storage / transport conditions:	Store / ship on wet ice (0-4°C) or with a frozen cold pack for up to 24 h after blood draw; follow UN Regulation 650 for packaging
3.5 Actions if requirements are not met:	Request new sample; test old sample, discard if no good. Note the potential for continued foci loss in warm samples.
4. Materials:	
4.1. Equipment and supportive materials:	• Deep 96-well plates and cling film; • 8 channel aspirator; • Single- and 8/12 channel micro-pipettors and tips • 37°C incubator and whirly mixer • Benchtop centrifuge with multiwell plate rotor • SuperFrostPlus glass slides and diamond cutter or multiwell slides with adhesive surface coating • Moist chamber (e.g. lidded plastic box with wet paper); • Tissues; cover slips, e.g. 22x50 mm^2. • Microcentrifuge and 1.5 ml tubes. • Fluorescence/phase contrast microscope.
4.2. Reagents:	• Phosphate buffered saline (PBS), without Mg & Ca; • BD™ Phosflow Lyse/Fix Buffer 5X, diluted to 1X with deionized H_2O, pre-warmed to 37°C; • 0.5% Triton-X100 in PBS; • Blocking solution (BS: 1x PBS, 1% BSA) • Mouse γ-H2AX antibody (e.g. Abcam, 1:500 in BS) • Fluorophore-conjugated 2nd antibody (e.g. Goat anti-mouse AlexaFluor 488, Invitrogen A-11029, 1:500 in blocking solution with 0.2 µg/ml DAPI or other suitable DNA counterstain); • Vectashield mounting medium; nail varnish.

5. Sample processing:	***All incubations are at ambient temperature unless stated otherwise***
5.1 Red blood cell lysis and fixation	(1) Aliquot 90 µl blood into a deep well plate (2 ml)[1] and add 1.8 ml of pre-warmed 1X Lyse/Fix buffer, (1:20). Cover with cling film, whirly-mix thoroughly and incubate for 14 minutes at 37°C. (2) Spin at 250 g for 5 minutes. Remove the supernatant, using an 8 channel aspirator and add 1.8 ml cold PBS. Mix, repeat step (2).
5.2 Sample transfer onto slide	(3) Spin at 250 g for 5 minutes. Remove the supernatant leaving ~ 50 µl. Resuspend the pellet and spread half of it onto a slide. Allow cells to adhere for ~15 min.[2]
5.3 Permeabilisation	(4) Incubate slide in 0.5%Triton-X100 for 5 min. (5) Drain slide and incubate in BS for 10min. (6) Inspect slides using a phase contrast microscope for suitable density, even distribution and integrity of cells.[3]
5.4 Antibody staining	(7) Remove excess liquid, apply 100µl γ-H2AX Ab solution and incubate in moist chamber for 40 min.[4] (8) Wash 3 x 2 min in blocking solution. (9) Remove excess liquid, apply 100µl 2nd Ab/DAPI solution and incubate in moist chamber in the dark for 30 min.[4] (10) Wash 3 x 2 min in PBS in the dark.
5.5 Mounting	(11) Drain excess liquid, add ~25 µl mounting medium and apply a cover slip. (12) Inspect with a fluorescence microscope with at least 40x lens to confirm presence and 'scorability' of fluorescently stained cells. (13) Store slides at 4°C in the dark.[5]
5.6 Notes	[1]Instead of deep-well plates, 2 ml microcentrifuge tubes can also be used. [2]25 µl suffice to cover a ~100mm^2 area on the slide. Smaller volumes should be used in combination with smaller slide areas, e.g. on multi-well slides. Avoid complete drying of the sample. Keep remaining cells on ice or at 4°C as backup, in case something goes wrong. [3] Make sure cells form a monolayer. At high densities cells can form multiple layers on top of each other which complicates foci scoring, especially using unsupervised automated approaches. Also check for red cell debris which can cause non-specific fluorescence. [4]Avoid drying of the cell area, as that would result in non-specific staining. Adjust antibody volume to stained area. [5]For long-term storage, slides should be sealed with nail varnish and stored at 4ºC for weeks/months or at -20ºC for longer periods. Repeated freezing and thawing should be avoided.