1. Aim of the method:	Define standard procedures and conditions for obtaining gamma-H2AX-immunostained lymphocyte samples suitable for biological dosimetry based on microscopic foci scoring.
2. Background:	Immunofluorescence staining of DNA double-strand break-associated nuclear foci in blood lymphocytes enables rapid detection of recent radiation exposure in unstimulated blood lymphocytes.
3. Sample requirements:	
3.1 Nature of sample	Aliquots of 2 ml human blood, X-irradiated and diluted 1:1 with RPMI1640 medium + 10% heat-inactivated FCS[1]; incubated for 2 h and 24 h at 37°C post irradiation.
3.2 Container:	Blood collection tubes with lithium heparin anti-coagulant
3.3 volume	2 ml blood + 2 ml medium per sample
3.4 Transport conditions:	Ship on wet ice (0-4°C) or with a frozen cold pack, temperature logger and dosimeter chip; 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 signal loss in warm samples.
4. Materials:	
4.1. Equipment and supportive materials:	• Cytospin with filter paper and slide holder, e.g. Shandon; • Benchtop centrifuge and 15 ml tubes; • Microcentrifuge and 1.5 ml tubes; • Microscopy slides with surface coating to enhance cell adhesion, e.g. SuperFrostPlus; • Fluorescence/phase contrast microscope with at least x40 lens; • Moist chamber (e.g. lidded plastic box with wet paper); • Coplin jars; micro-pipettors and tips; tissues, cover slips, e.g. 24x60 mm^2; Parafilm stripes.
4.2. Reagents:	• Phosphate buffered saline (PBS), without Mg & Ca; • Deionized H_2O; • 0.25% Triton-X100, 0.1% glycine in PBS; • 2% formaldehyde (FA), acid free, in PBS; • Blocking solution (BS; 1xPBS, 1% BSA, 0.1% Tween20); • Anti γ-H2AX antibody (e.g. Millipore 05-636, 1:500 in BS); • Fluorophore-conjugated 2nd antibody (e.g. AlexaFluor488 goat anti-mouse, Invitrogen A-11029, 1:500 in BS); • Mounting medium with antifade and fluorescent DNA counterstain: e.g. Vectashield with DAPI; • Ficoll-based density gradient cell separation medium for human lymphocyte isolation, e.g. Histopaque 1.077;
5. Sample processing:	***All incubations are at ambient temperature unless stated otherwise***
5.1 Isolation of lymphocytes	(1) Layer 4 ml diluted blood carefully[2] onto 4 ml Histopaque in 15 ml tube

	(2) Spin at 1200 g for 5 min without brake[4] and transfer buffy coat cell layer into a 15 ml tube. (3) Add 10ml PBS and swirl briefly; spin 5 min at 400 g; discard supernatant; repeat step (3) once.
5.2 Cytospin	(4) Resuspend pellet in 2 ml PBS. Adjust cell concentration to cytospin 60-100 000 cells onto a slide for 1 min at 500 RPM.[5]
5.2A [Alternative: manual spotting]	*(4A) [Alternative: Resuspend pellet in 0.1 ml PBS and spread 25 µl onto a slide. Allow cells to adhere for ~15 min.][6]*
	(5) Inspect slides using a phase contrast microscope for suitable density, even distribution and integrity of cells.[7]
5.3 Fixation and permeabilisation	(6) Fix cells with 2% FA for 5 min. *[Optional: Store slides overnight in 0.5% FA/PBS at 4°C]* (7) Extract cells in 0.25%Triton-X100, 0.1% glycine/PBS for 5 min[8] (8) Drain and incubate slides in BS for 10min.
5.4 Antibody staining	(9) Remove excess liquid, apply 90µl γ-H2AX Ab solution, cover with Parafilm and incubate in moist chamber for 45 min.[9] (10) Remove Parafilm with forceps and wash 3 x 2 min in BS. (11) Remove excess liquid, apply 90µl 2nd Ab solution, cover with Parafilm and incubate in moist chamber in the dark for 30 min.[9] (12) Remove Parafilm and wash 3 x 2 min in PBS in the dark.
5.5 Mounting	(13) Drain excess liquid, add 18µl mounting medium with DNA counterstain[10] and apply a cover slip. (14) Store slides at 4°C until anaylsis.[11] (15) Score foci per cell in random fields of view, using at least an x40 lens. Record results for 20, 30 and 50 scored cells.
5.6 Notes	[1]This dilution step reduces haemolysis and enhances lymphocyte recovery. Heat inactivated FCS prevents lymphocytes reacting to 'complement'. [2]Tilt tube and let diluted blood run slowly along the tube wall to avoid any mixing. [3]Specific isolation of the T-cell subset may reduce uncertainties in the dose estimate, caused by heterogeneous foci formation in different lymphocyte subsets. [4]Active braking at the end of the centrifugation spreads out the buffy coat layer. [5]Exact cell numbers, volumes and centrifugation time/RPM depend on the specifications of the cytospin equipment. Keep remaining cells on ice or at 4°C as backup, in case something goes wrong. [6]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 a backup, in case something goes wrong. [7]Especially 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. [8]Glycine captures unbound FA, thus eliminating the need for additional washing steps. [9]Avoid drying of the cell area, as that would result in non-specific staining. [10]DNA counterstain can alternatively be added to the 2nd Ab solution to reduce non-specific background fluorescence. [11]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.