Micronuclei Assay

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Abstract

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Protocol

Blood Collection

Step 1.
Colect blood into heparinized blood collection tubes (for instance Li-heparinized tubes, Vacuette, Mundelein, IL, USA)

Blood Collection

Step 2.
Blood can be stored at room temperature (22 ± 1ºC) up to 2 h prior lymphocyte separation.

Peripheral blood mononuclear cell (PBMC) separation

Step 3.

Peripheral blood mononuclear cell (PBMC) separation

Step 4.
Warm Histopaque-1077 density gradient medium (cat. No. 10771, Merck, Kenilworth, NJ, USA) at 37°C water bath (NE2-8D Unstirred digital water bath, Nickel-Electro Ltd., Weston-super-Mare, UK) for 30 min before use.

Peripheral blood mononuclear cell (PBMC) separation

Step 5.
In the laminar flow box, pour Histopaque-1077 medium (37°C) into a 15 conical tubes (TPP Techno Plastic Products AG, Trasadingen, Switzerland). For volumes, see 3).

Peripheral blood mononuclear cell (PBMC) separation

Step 6.
Overlay blood by gently pipetting onto Histopaque-1077 medium using ratio of approximately 1:1. *IMPORTANT:* To obtain good separation, both blood and Histopaque layers must be clearly separated before centrifugation.

Peripheral blood mononuclear cell (PBMC) separation

Step 7.
Spin at 400 g (BR4i multifunction centrifuge, Thermo Electron Corp., Waltham, MA, USA) for 30 min at 37 °C after carefully balancing the tubes. *IMPORTANT:* brakes off!

Peripheral blood mononuclear cell (PBMC) separation

Step 8.
While removing the tubes from the centrifuge do not disturb the layering.

Peripheral blood mononuclear cell (PBMC) separation

Step 9.

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Collect the PBMC with sterile pasteur pipette at the interface of Histopaque-1077 medium and plasma into new 15 mL conical tubes. *IMPORTANT:* Try to avoid apsiring Histopaque-1077 medium as much as possible.

Peripheral blood mononuclear cell (PBMC) separation

Step 10.

Wash the PBMC by adding RPMI-1640 culture medium (cat. No. **R8758,** Merck) to make up 10 mL.

Peripheral blood mononuclear cell (PBMC) separation

Step 11.

Spin at 280 g for 5 min at 37 °C.

Peripheral blood mononuclear cell (PBMC) separation

Step 12.

Discard the supernatant, resuspend the pellet and wash again by adding RPMI-1640 to make up 10 mL.

Peripheral blood mononuclear cell (PBMC) separation

Step 13.

Spin at 280 g for 5 min at 37 °C.

Peripheral blood mononuclear cell (PBMC) separation

Step 14.

Discard the supernatant, resuspend the pellet and add 1 ml RPMI 1640 culture medium.

Cultivation of PBMC

Step 15.

Cultivation of PBMC

Step 16.

Measure the cell concentration and viability using Counting chamber with spring clips (BRAND GMBH + CO KG, Wertheim, Germany). For that, mix 10 µL of the PBMC with trypan blue solution at 1:1 ratio and wait for at least 30 seconds before counting.

Cultivation of PBMC

Step 17.

Resuspend the cells in RPMI-1640 culture medium supplemented with with 20% fetal bovine serum (PAA Laboratories GmbH, Austria), 2 mmol/l glutamine, 100 UI/ml penicillin and 0.1 mg/ml streptomycin (all from Merck) at 1.0×10^6 cells/ml.

Cultivation of PBMC

Step 18.

PBMC are then stimulated by Phytohemaglutinin (cat. No. L1668, Merck) at a concentration of 10 µg/ml.

Cultivation of PBMC

Step 19.

After stimulation, incubate at 37°C, 5% CO_2 in a humidified atmosphere (Galaxy 170R, Eppendorf AG, Hamburk, Germany) with loose lids.

Cultivation of PBMC

Step 20.

Add cytochalasin B (cat. No. C6762, Merck) 44 h after PHA stimulation at a concentration of 6 ug/ml to block cells at cytokinesis. PBMC are then incubated at 37°C, 5% CO_2 in a humidified atmosphere with loose lids for next 28 h.

Harvest, fixation and slide preparation of PBMC

Step 21.

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Harvest, fixation and slide preparation of PBMC

Step 22.

After the incubation, spin the cells at 280 g for 10 min at 37°C.

Harvest, fixation and slide preparation of PBMC

Step 23.

Discard the supernatant and resuspend the pellet.

Harvest, fixation and slide preparation of PBMC

Step 24.

Wash the cells once with 5 mL of ice-cold fixative solution containing methanol:acetic acid (3:1) with 1% formaldehyde (all from Merck). *IMPORTANT:* The fixative should be added by slow dripping while vortexing the cells to prevent forming of clumps.

Harvest, fixation and slide preparation of PBMC

Step 25.

Spin at 200 g for 10 min at 4°C.

Harvest, fixation and slide preparation of PBMC

Step 26.

Discard the supernatant and resuspend the cells in 5 mL of ice-cold fixative, this time without formaldehyde.

Harvest, fixation and slide preparation of PBMC

Step 27.

Spin again.

Harvest, fixation and slide preparation of PBMC

Step 28.

Discard the supernatant and resuspend the cells in 5 mL of ice-cold fixative (without formaldehyde).

Harvest, fixation and slide preparation of PBMC

Step 29.

Spin again.

Harvest, fixation and slide preparation of PBMC

Step 30.

Discard all but 0.5 mL of the supernatant and resuspend the cells in the remaining fluid. *NOTE:* Fixed cells can be stored at this point at -20°C.

Harvest, fixation and slide preparation of PBMC

Step 31.

Drip the resuspended cells (80 µL) using a pipette onto a clean, grease free slide that has been previously stored in a freezer from a height of at least 10 cm.

Harvest, fixation and slide preparation of PBMC

Step 32.

Allow the slides to dry at room temperature.

Staining

Step 33.

Staining

Step 34.

Stain air-dried slides using 4% Giemsa stain (cat. No. 32884, Merck) for 8 min.

Staining

Step 35.

Rinse in tap water and allow to dry at room temperature.

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Sample evaluation

Step 36.

Sample evaluation

Step 37.

Evaluate stained samples using microscope (BX-51, Olympus, Tokyo, Japan) at 400fold original magnification.

Sample evaluation

Step 38.

Evaluate a total of 1000 binucleated (BN) cells for the frequency of micronuclei (MN).

Criteria for selecting binucleated cells (Fenech et al. 2000)

Step 39.

Criteria for selecting binucleated cells (Fenech et al. 2000)

Step 40.

The cells should be BN.

Criteria for selecting binucleated cells (Fenech et al. 2000)

Step 41.

The two nuclei in a BN cell should have intact nuclear membranes and be situated within the same cytoplasmic boundary.

Criteria for selecting binucleated cells (Fenech et al. 2000)

Step 42.

The two nuclei in a BN cell should be approximately equal in size, staining pattern and staining intensity.

Criteria for selecting binucleated cells (Fenech et al. 2000)

Step 43.

The two nuclei within a BN cell may be attached by a fine nucleoplasmic bridge wich is no wider than one-fourth of the largest nuclear diameter.

Criteria for selecting binucleated cells (Fenech et al. 2000)

Step 44.

The two main nuclei in a BN cell may touch but ideally should not overlap each other. A cell with two overlapping nuclei can be scored only if the nuclear boundaries of each nucleus are distinguishable.

Criteria for selecting binucleated cells (Fenech et al. 2000)

Step 45.

The cytoplasmic boundary or membrane of a binucleated cell should be intact and clearly distinguishable from the cytoplasmic boundary of adjacent cells.

Criteria for scoring MN (Fenech et al. 2000)

Step 46.

MN are morphologically identical to but smaller than nuclei with the following characteristics:

Criteria for scoring MN (Fenech et al. 2000)

Step 47.

The diameter of MN usually varies between 1/16th and 1/3rd of the mean diameter of the main nuclei, which corresponds to 1/256th and 1/9th of the area of one of the main nuclei in a BN cell, respectively.

Criteria for scoring MN (Fenech et al. 2000)

Step 48.

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MN are usually round or oval in shape, non-refractile and therefore, be readily distinguishable from artefact.

Criteria for scoring MN (Fenech et al. 2000)

Step 49.

MN are not linked or connected to the main nuclei.

Criteria for scoring MN (Fenech et al. 2000)

Step 50.

MN may touch but not overlap the main nuclei and the micronuclear boundary should be distinguishable from the nuclear boundary.

Criteria for scoring MN (Fenech et al. 2000)

Step 51.

MN usually have the same staining intensity as the main nuclei but occasionally staining may be less or more intense.

Criteria for scoring MN (Fenech et al. 2000)

Step 52.

The diameter of MN in human lymphocytes usually varies between 1/16 and 1/3 of the mean.

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