A common European approach to the regulatory testing of nanomaterials

Standard Operating Procedure

Title	***Micronucleus assay using Flow Cytometry***
Subtitle	High Throughput Assay
NANoREG Work package/task:	WP5 / Task 5.06
Owner and co-owner(s)	Ricard Marcos, UAB
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1	2017/07/10	Harmonisation according NANoREG template for SOPs
2		
3		
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Notes on protocol

1. First of all it is necessary to know the doubling time of the cells used (1pd) to adjust the ending time of the culture before start the MN study.

2. To analyze MN frequency only concentrations producing survival rates higher than 40%, compared to the untreated control, must be used.

1 Materials

- PBS + 2% FBS (inactivated)
- EMA Dye: 0.125 mg/mL in PBS + 2% FBS
- Lysis buffer 1: (use deionized water) 0.5 mg/mL NaCl + 1 mg/mL sodium citrate + 0.3 µL/mL IGEPAL, 1 mg/mL RNase A and 0.2 µM Sytox green
- Lysis buffer 2: (use deionized water) 85.6 mg/mL sucrose, 15 mg/mL citric acid and 0.2 µM Sytox green

2 Procedure

1) Seed 0.3 million cells in multiwell plates (6 wells).
2) 24 h after seeding, start the treatment with DEB (0/0.05 µg/mL). Treat for 48 h or 72 h, depending on the cell line (calculate at least 1 pd).
3) The day of finish culturing, trypsinize and count the untreated cells with the counter-coulter to know if they are growing and have divided once.
4) Pass the cells into FACS tubes.
5) Centrifuge at 800 rpm for 8 min, aspirate supernatant and resuspend the pellet by gently tapping the tube.
6) Prepare a mixture containing 100 µL PBS + 2% FBS and 25 µL EMA dye per tube. Add a total of 125 µL of this solution to each tube.
7) Place the tubes on ice (2 cm deep) and irradiate with a 60 W visible light bulb that's 30 cm away from the tubes for 20 min (photo-activation step).
8) After this step PROTECT THE SAMPLES FROM ALL LIGHT SOURCES AT ALL TIMES.
9) Add 3 mL of cold PBS + 2% FBS.
10) Centrifuge at 800 rpm for 8 min, aspirate the supernatant leaving about 50 µL and resuspend the pellet by gently tapping the tube.
11) Leave the samples at room temperature for 20 min.
12) Slowly add 250 µL of lysis buffer 1 (about 15 seconds per sample) and immediately vortex for 5 seconds at medium speed.
13) Keep the samples at room temperature for 1 h.
14) Vigorously inject 250 µL of lysis buffer 2 into each sample and immediately vortex for 5 seconds at medium speed.
15) Leave 30 min at room temperature.
16) Store at 4 °C until the analysis on the cytometer (up to two days).
17) Analyse with the cytometer capturing 20,000 nuclei.