Standard Operating Procedure @ CNR- IREA

PROTOCOL	**Cell cycle progression by flow cytometer in SH-SY5Y cells**
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1. Purpose

This procedure describes the materials and the protocol used for flow cytometric analysis of cell cycle with propidium iodide staining in SH-SY5Y cells.

2. Background

Propidium iodide (PI) is a fluorescent dye intercalating into the base pairs of double-stranded DNA. PI is used to quantify DNA content in permeabilized cells by flow cytometry. Cell cycle analysis consists in quantifying the percentage of cells in each stage of the cell cycle (G0/G1, S and G2/M).

3. Procedure

3.1. Equipments

- Cell culture incubator (Thermo Scientific Forma, Model 311)
- Refrigerated centrifuge (Heraeus Sepatech Minifuge RF)
- Automated cell counter (Luna II)
- 4°C refrigerator
- FACSCalibur flow cytometer (BD Biosciences) equipped with 488 nm laser

3.2. Materials

- 35 mm cell culture dish (Corning, cod. 430165)
- 5 mL polystyrene round bottom FACS tube (Falcon, cod. 352052)

3.3. Reagents

- Sodium chloride solution 0.9% (Galenica senese, cod A029874385) is stored at room temperature (RT)
- MMC (Mitomycin-C; Sigma, cod. 1001017941): 0.25 mg/ml stock solution is prepared in sodium chloride solution. Aliquots of 500 µl are prepared and stored in the dark at -20°C
- DMEM (Dulbecco's Modified Eagle Medium), supplemented with 4.5 g/L glucose, sodium pyruvate and sodium bicarbonate (Microgem, cod. AL007) is stored at +4°C
    - For complete medium, supplement DMEM with: 10% heat inactivated Fetal Bovine Serum (FBS; Microgem, cod. RM10432), 2 mM GlutaMAX™ Supplement (Gibco, cod. 35050-038), 1X Penicillin-Streptomycin solution (Himedia, cod. A001), and store at 4°C
- 1X PBS (Dulbecco's Phosphate Buffered Saline) w/o Calcium w/o Magnesium (Microgem, cod. TL1006) is stored at +4°C
- Trypsin – EDTA 1X in solution w/o Calcium w/o Magnesium w/ Phenol Red (Microgem, cod. L0930) is stored at -20°C
- Trypan blue stain, 0.4% (Logos Biosystems, cod. TB0BJC2301) is stored at RT
- Triton X-100 solution (Sigma, cod. 93443) is stored at RT
- Sodium citrate dihydrate (Baker, cod. 15598154): 0.75 M stock solution is prepared in H_2O_2, pH 8 and stored at RT
- PI, 1mg/ml (Sigma, cod. P4864) is stored at +4°C
    - For PI solution: 50 µg/ml, 33 mM sodium citrate and 0.1% Triton X-100. It is stored at +4°C

3.4. Experimental procedure

The following procedure has been optimized for SH-SY5Y human neuroblastoma cells (ATCC, Cat. No. CRL2266, Rockville, MD, USA). Procedure for cell maintenance is detailed in Annex 1.

$1x10^6$ cells are seeded in 3 ml complete medium in a 35 mm cell culture dish and the assay is performed after 72 h of growth.

3.4.1. Positive control

Cells treated for 16 h with 1 µg/ml MMC are used as positive control.

3.4.2. Sample preparation

1) Collect cell growth medium into FACS tube
2) Wash the adherent cells with PBS (1 ml) and detach by 3 min trypsin treatment (300 µl) at 37°C
3) Collect the cells by using the growth medium and add cold PBS (500 µl)
4) Centrifuge (4°C, 1200 RPM, 5 min), and remove the supernatant
5) Resuspend the cell pellet with DMEM complete medium (1 ml)
6) Count the cells by trypan blue stain
7) Transfer 500000 cells into a clean FACS tube
8) Add cold PBS (2 ml), centrifuge (4°C, 1200 RPM, 5 min) and remove the supernatant. Repeat this step once
9) Resuspend the cell pellet with 500 µl of cold DMEM base medium and 500 µl of cold PI solution
10) Mix well and vortex
11) Incubate for 30 min at 4°C
12) Analyze by flow cytometer

Note: be sure that steps 7 to 10 are performed in the dark.

3.4.3. Sample acquisition

CellQuest software is used for sample acquisition and data storage.

For each sample, 25000 events are acquired. Forward scatter (FSC) and side scatter (SSC) dot plots are selected to identify the cell population. FL2 channel is selected to detect PI fluorescence in the linear scale.

The following graphs are displayed:

1° Dot Plot: Acquisition, FSC (x-axis) and SSC (y-axis)

2° Dot Plot: Acquisition, FL2W-1024 (x-axis) and FL2-A (y-axis)

1° histogram: Acquisition, FL2-A

2° histogram: Acquisition, FL2-H

Note:

- adjust the sensitivity of photomultiplier tubes for PI staining such that the G0/G1 (2n ploidy) and G2/M (4n ploidy) are centered, respectively, at 200 and 400 (arbitrary units) on the X-axis
- a S-shaped population should be visible in the 2° Dot Plot

3.4.4. Sample analysis

FlowJo software (TreeStar, OR, USA) is used for the analysis. Data are expressed as the relative percentage of cells in different stages of the cell cycle.