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TUNEL Assay and Cell Cycle distribution with PI staining (Nuclear apoptosis analysis and cell cycle distribution)

1. Test principle

Apoptotic cells can be detected by terminal deoxynucleotidyl transferase (TdT) – mediated dUTP-biotin nick-end labeling (TUNEL).

Cells are first fixed with a crosslinking agent (such as paraformaldehyde) to prevent the loss of low molecular weight DNA fragments. To make the DNA accessible to the labeling enzyme, the cell membranes are permeabilized. Biotinylated nucleotides are incorporated into the 3'-OH ends of the DNA fragments by TdT. When attached to DNA strand breaks in the form of poly-BrdU, this deoxynucleotide can be detected with a FITC-conjugated anti-BrdU Ab. The fluorescein-stained cells are subsequently visualized using flow cytometer.

Labeling of DNA strand breaks in this procedure, which utilizes fluorescein-conjugated anti-BrdU Ab, can be combined with staining of DNA by a fluorochrome of another color (Propidium Iodide for cell cycle distribution).

2. Specimen

Cells in suspension, from whole blood, bone marrow or cell culture

3. Materials and reagents

- 5ml Falcon polypropylene Round-Bottom test tubes (12x75 mm)
- cooling centrifuge
- sterile Phosphate-buffered saline (PBS)
- 4% Paraformaldehyde (Sigma Chemical) in PBS, pH 7.4 (primary fixative)
- 70 % ethanol (second fixative)
- 5x TdT reaction buffer (see recipe)
- 2 mM BrdUTP (Sigma) in 50 mM Tris.Cl, pH 7.5
- TdT in storage buffer (Roche Diagnostics), 25 U in 1 μ L
- 10 mM cobalt chloride (CoCl₂; Roche Diagnostics)
- Rinsing buffer: PBS with 0.1 % (v/v) Triton X-100 and 0.5 % (w/v) BSA
- FITC- conjugated anti-BrdUTP Mab in PBS (see recipe)
- PI staining buffer: PBS with 5 μ g/ml PI and 200 μ g/ ml DNase-free RNase

4. Controls:

- Positive control: cells with 10 mg/ml DNase I for 10 min at room temperature (or other drug treated unhealthy cells)
- Negative control: TdT enzyme was not added
- Cells stained with dUTP-FITC alone for flow cytometry setup control
- Untreated cells with no dUTP-FITC and only PI added for flow cytometry setup control

5. Procedure

1. Prepare cells in appropriate manner to get approximately 10^6 cells in each test tube.
 2. 1×10^6 washed cells were fixed with 4% paraformaldehyde in PBS for 30 min. at room temperature.
 3. Centrifuge 5 min at 200 xg, 4 °C, resuspend cell pellet in 4 mL PBS, centrifuge 5 min at 200 xg, 4 C, resuspend cell pellet in 0.5 mL PBS
 4. Add 4 mL ice-cold 70 % ethanol into the tube while vortexing.
The cells can be stored several weeks in ethanol at -20 °C.
 5. Centrifuge 5 min at 200 x g, 4 °C, remove ethanol, and resuspend cells in 5 mL PBS. Repeat centrifugation.
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6. Resuspend the pellet (10^6 cells) in 50 μ L of a solution that contains:
 - 10 μ L 5 x TdT reaction buffer
 - 2.0 μ L 2 mM BrdUTP stock solution
 - 0.5 μ L TdT in storage buffer (12.5 U final)
 - 5 μ L 10 mM CoCl_2 solution
 - 32.5 μ L dH_2O .
7. Incubate cells in this solution 40 min at 37 °C.
Alternatively, incubation can be carried out overnight at 22 C to 24 C.
8. Add 1.5 mL rinsing buffer and centrifuge 5 min at 200 x g, room temperature.
9. Resuspend cells in 100 μ L FITC-conjugated anti-BrdU MAb solution.
10. Incubate 1 hr at room temperature or overnight at 4 C. Add 2 mL rinsing buffer and centrifuge 5 min at 200 x g, room temperature.
11. Resuspend cell pellet in 1 mL PI staining solution containing RNase. Incubate 30 min at room temperature in the dark.
12. Measure cell fluorescence on the flow cytometer.

6. Recipe

TdT reaction buffer; 5x

- 1 M potassium (or sodium) cacodylate
- 125 mM Tris-Cl, pH 6.6
- 0.125 % bovine serum albumin (BSA)
- May be stored for weeks at 4 °C

FITC-conjugated anti-BrdU Mab solution

- 100 μ l PBS
- 0.3 μ g FITC-conjugated anti-BrdU MAb (Becton Dickinson)
- 0.3 % (v/v) Triton X-100
- 1 % (w/v) BSA
- Prepare fresh before use

RNase A solution

Dissolve 50 mg RNase A (Sigma, R-4875) in 50 ml of PBS and adjust to 0.1% Tween-20. Place solution into a 95°C waterbath for 30 min. Allow the solution to cool on ice for 1 hr. Remove precipitate by filtering through a 0.2 μ m filter. Solution can be stored for up to 6 months at 4°C or in aliquots be frozen at -20°C.

Propidium iodide (PI) solution

Dissolve PI (Sigma, P 4170) in dH_2O at 1 mg/ml. Store aliquots at -20°C for up to 2 years. Aliquots that are frequently used can be stored at 4°C for up to 2 months. Discard solutions of PI that have been exposed to room temperature for more than 48 hr, or if they appear dark red.

7. Note:

- PI is a nucleic acid-specific, red-fluorescent dye. It is also a suspected carcinogen, so employ appropriate safety precautions when working with this dye. This dye will bind to both DNA and RNA, however, treatment of the ethanol-fixed cells with RNase A, as described above, will digest all of the RNA so that the PI will fluorescently label the DNA, specifically.
- Many vendors sell kits with pre-prepared buffers and reagents. Alternatively, individual reagents may be purchased separately. The above protocol is a general guide and individual kits may vary in the amounts of reagents used.