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APO-BRDU *IN SITU* DNA FRAGMENTATION ASSAY KIT

Code: 310-BV-1FA1 ***Lot No.:*** ***Exp.:*** 6 months from date of dispatch

Introduction: Internucleosomal DNA fragmentation is a hallmark of apoptosis in mammalian cells. ImmunoKontact's Apo-BrdU *In Situ* DNA Fragmentation Assay Kit provides complete components including positive and negative control cells for conveniently detecting DNA fragmentation by fluorescence microscopy or flow cytometry. The kit utilizes Br-dUTP (bromolated deoxyuridine triphosphate nucleotides) which is more readily incorporated into DNA strand breaks than other larger ligands (e.g. fluorescein, biotin or digoxigenin). The greater incorporation gives rise to a brighter signal when the Br-dUTP sites are identified by a fluorescein labelled anti-BrdU monoclonal antibody

Kit Contents*:*

Components	Colour code	Volume	Store temp °C
Positive Control Cells	Brown cap	5ml	-20
Negative Control Cells	Natural cap	5ml	-20
Wash Buffer	Blue cap	120 ml	+4
Reaction Buffer	Green cap	0.6 ml	+4
TdT Enzymes	Yellow cap	45 ul	-20
Br-dUTP	Violet cap	0.48 ml	-20
Rinse Buffer	Red cap	120 ml	+4
Anti-BrdU-FITC Antibody	Orange cap	0.3 ml	+4
PI/RNase Staining Buffer	amber bottle	30 ml	+4

II. APOBRDU ASSAY PROTOCOL FOR CULTURED CELLS:

A. Cell Fixation

1. Induce apoptosis in cells by desired method. Concurrently incubate a control culture without induction.
2. Pellet 1-5 x 10^6 cells and resuspend in 0.5 ml of PBS.
3. Fix the cells by adding 5 ml of 1% (w/v) paraformaldehyde in PBS and place on ice for 15 minutes.
4. Centrifuge the cells for 5 min at 300 x g and discard the supernatant.
5. Wash cells in 5 ml of PBS and pellet the cells by centrifugation. Repeat the wash and centrifugation step once.
6. Resuspend the cells in 0.5 ml of PBS.
7. Add the cells to 5 ml of ice-cold 70% (v/v) ethanol. Let cells stand for a minimum of 30 min (or overnight if you prefer) on ice or in the freezer.
8. Store the cells in 70% (v/v) ethanol at -20^{o}C until use. Cells can be stored at -20^{o}C for several days before use.

B. Detection by Flow Cytometry and Fluorescence Microscopy:

The procedures can be used for both control cells and your testing cells.

1. Resuspend the fixed cells by swirling the vials. Remove 1 ml aliquots of the cell suspension (~1 x 10^6 cells per ml) and place in 12 x 75 mm tubes. Centrifuge (300 x g) for 5 min and carefully remove the ethanol by aspiration.
2. Resuspend the cells with 1 ml of **Wash Buffer** (blue cap). Centrifuge as before and remove supernatant carefully by aspiration.
3. Repeat the washing step once (step 2).
4. Resuspend in 50 ul of the **DNA Labelling Solution** prepared as below:

DNA Labelling Solution	1 assay	10 assays
TdT Reaction Buffer (green cap)	10 ul	100 ul
TdT Enzyme (yellow cap)	0.75 ul	7.5 ul
Br-dUTP (violet cap)	8 ul	80 ul
ddH_20	32.25 ul	322.5 ul
Total Volume	51 ul	510 ul

5. Incubate the cells in the **DNA Labeling Solution** for 60 min at 37°C. Shake cells every 15 min to resuspend.
6. Add 1 ml of **Rinse Buffer** (red cap) to each tube and centrifuge for 5 min. Remove supernatant by aspiration.
7. Repeat the rinsing step once (step 6).
8. Resuspend cells in 0.1 ml of the **Antibody Solution** prepared as below:

Antibody Solution	1 assay	10 assays
Anti-BrdU-FITC Antibody (orange cap)	5 ul	50 ul
Rinse Buffer (red cap)	95 ul	950 ul

9. Incubate the cells with the **Antibody Solution** in the dark for 30 min at room temperature.
10. Add 0.5 ml of **Propidium Iodide/RNase A Solution** (amber bottle).
11. Incubate the cells in the dark for 30 min at room temperature.
12. Analyze the cells by fluorescence microscopy (apoptotic cells show green staining over an orange-red PI counter-staining) or flow cytometry. Cells should be analyzed within 3 hours of staining.

IV. APO-BRDU ASSAY PROTOCOL FOR TISSUE SECTIONS:

A. Tissue Section Preparations:

The protocol describes the preparation of formalin-fixed, paraffin-embedded tissue section mounted on glass slides. For information on fixing and embedding techniques, see Ben-Sasson *et al*., (Methods Cell. Biol. 46:29-39, 1995). Most steps are performed in Coplin Jars.

Note: If using fresh-frozen tissue sections, proceed directly to step 7.

1. Remove paraffin by immersing slides in a Coplin jar containing fresh xylene. Incubate at room temperature for 5 minutes.
2. Repeat in a second Coplin jar containing fresh xylene.
3. Immerse the slides in a Coplin Jar containing 100% ethanol and incubate at room temperature for 5 min.
4. Rehydrate the slides by sequential 3-min, room temperature incubations in Coplin jars containing:
    a. 100% ethanol
    b. 95% ethanol
    c. 85% ethanol
    d. 70% ethanol
    e. 50% ethanol
5. Immerse the slides in a Coplin jar containing 0.85% NaCl and incubate at room temperature for 5 min.
6. Immerse the slides in a Coplin jar containing PBS and incubate at room temperature for 5 minutes.
7. Fix the slides by immersing them in a Coplin jar containing fresh 4% formaldehyde/PBS, and incubate at room temperature for 15 min.
8. Wash the slides by immersing them in a Coplin jar containing PBS, and incubate at room temp for 5 min.
9. Transfer to another Coplin jar containing PBS, and incubate at room temperature for 5 min.

10. Allow the liquid to drain thoroughly and place slides on a flat surface.
11. Prepare 20 ug/ml of Proteinase K Solution (combine 2 ul of 10 mg/ml Protease K and 998 ul of 100 mM Tris-HCl, pH 8.0, 50 mM EDTA) and cover each section with 100 ul of it. Incubate at room temperature for 5 min.
12. Immerse the slides in Coplin jar containing PBS, and incubate at room temperature for 5 min.
13. Transfer the slides to a Coplin jar containing 4% formaldehyde/PBS and incubate at room temperature for 5 minutes.
14. Wash the slides by immersion in Coplin jar containing PBS, and incubate at room temperature for 5 min.

B. ***Detection by Fluorescence Microscopy:***

a. Remove slides from PBS and tap gently to remove excess liquid. Cover the cells in 100 ul of Wash buffer (blue cap).
b. Use forceps, gently place a piece of plastic coverslip on top of the cells to evenly spread the liquid, incubate for 5 min. Remove plastic coverslip and gently tap the slides to remove excess liquid.
c. Repeat step 2. Carefully blot dry around the edges with tissue paper.
d. Gently place 50 ul of the DNA Labeling Solution (prepared as in Section IIIB, Step 4) on the cells.
e. Use forceps, gently place a piece of plastic coverslip on top of the cells to evenly spread the liquid.
f. Place the slides in a dark, humidified 37^{o}C incubator for 60 minutes.
   Note: Ensure high humidity by placing wet paper towels in the bottom of the dry incubator.
g. Using forceps, remove the plastic coverslips. Rinse the slides to a fresh Coplin jar filled with PBS for 5 min.
h. Repeat step 7. Carefully blot dry around the edges with tissue paper.
i. Place 100 ul of the Antibody Solution (Prepared as in Section IIIB, step 8).
j. Use forceps, gently place a piece of plastic coverslip on top of the cells to evenly spread the liquid.
k. Incubate the cells with the antibody solution in a humidified incubator for 30 min at room temperature.
l. Carefully remove the solution from slides. Add 100 ul of Propidium Iodide/Rnase A solution (amber bottle).
m. Use forceps, gently place a piece of plastic coverslip on top of the cells to evenly spread the liquid.
n. Incubate the slides in the dark in a humidified incubator for 30 min at room temperature.
o. Wash the cells by transferring the slides to a fresh Coplin jar filled with ddH_2O and incubate at room temperature for 5 min.
p. Repeat Step 15.
q. [Optional] Add a drop of anti-Fade solution and cover the treated portion of the slide with a glass coverslip.
r. [Optional] Seal the edges of the coverslip with rubber cement or clear nail polish.
s. View slides as soon as possible. Apoptotic cells will exhibit strong nuclear green fluorescence. All cells should be stained with PI and exhibit strong red counter staining.