THIS PROTOCOL WAS DEMONSTRATED BY DR. N.P. SINGH AT THE WORKSHOP ON COMET ASSAY HELD DURING FEBRUARY 7-11, 2003, AT INDUSTRIAL TOXICOLOGY RESEARCH CENTRE, LUCKNOW, INDIA

Summary protocol for assessment of APOPTOSIS

Apoptosis by DNA diffusion assay (Narendra Singh, in "Methods in Molecular Medicine (Chemosensitivity)", ed: Rosalyn Blumenthal, 2003, Humana Press, Totowas, NJ, USA)

Solutions

Agarose (MADE 24 HR PRIOR TO USE)

1. 0.7% high resolution agarose 3:1 in PBS without Ca++ Mg++, with 20 mM tris
2. 2% SFR agarose in PBS without Ca++ Mg++, with 20 mM tris

Lysing and alkaline Solution (room temp)

1.25 M NaCl
0.01% Sodium N-Lauroyl Sarcosine (SLS)
1 mM tetrasodium salt of EDTA
5 mM Tris-HCl (pH10)
0.2% DMSO added fresh
300mM NaOH added fresh

Neutralizing /DNA precipitating Solution

50% ethanol
1 mg/ml spermine
20 mM of Tris-HCl (pH 7.4)

Staining Solution

0.25 micromolar YOYO
2.5% DMSO
0.5% sucrose

Slide Preparation

1. Base Layer first coat: 50 microliters agarose, smeared on slide and air-dried.
2. Prepare cell-agarose suspension. [Low light]
3. Cell Layer: 50 microliters of cell-agarose suspension, cover with cover glass. [Low light]
4. Remove cover glass. [Low light]
5. Third layer, 200 microliters SFR agarose, cover with cover glass.
6. Keep protected from light until ready to put into lysing solution.
7. Remove cover glass just before lysis.

Slide Processing

1. Prepare fresh lysing and alkaline solution
2. Lyse in alkaline solution for 10 min at room temperature [low light]
3. Immerse slides in ***Neutralizing /DNA precipitating Solution*** for 30 min at room temperature. Repeat this step once more in fresh solution.
4. Allow slides to air-dry.
5. Store in slide boxes until ready to stain and analyze.

Staining

1. Apply 50 microliters of stain in 5 small, equally spaced droplets over the clear window area and spread it by using pipet tip but without touching the slide and cover with cover glass.
2. Analyze immediately.

Detailed protocol for assessment of APOPTOSIS:

Induction of Apoptosis in Leukocytes:
As a model for standardizing the DNA diffusion assay, apoptosis can be induced in whole blood leukocytes. Leukocytes undergo spontaneous apoptosis at 37 degrees C or room temperature (26 degrees C) in vitro. Three to 10 % of cells undergo apoptosis in 24 hr and 30-60% in 48 hr, depending on the age and nutrition status of the individual from whom the blood is collected.

Materials:

1. MGE slides (window size: 3cm × 1cm) (Erie Scientific Company, Portsmouth, NH, Cat. No. ES 370)
2. 3:1 agarose (Amresco, Solon, OH) or Normal Melting agarose (BRL Life Technologies Inc. Gaithersburg, MD, USA)
3. Agarose SFR (Amresco, Solon, OH)
4. Phosphate Buffered Saline (PBS) (Ca++, Mg++ free)(Hi Media Pvt. Ltd. India)
5. Sodium Chloride
6. Sodium Lauryl Sarcosinate (SLS)
7. Ethylene Diamine Tetra Acetic Acid (EDTA) Tetra sodium salt
8. Tris
9. Spermine
10. Ethanol
11. Dimethyl Sulphoxide (DMSO)
12. YOYO -1 Dye
13. Sucrose
14. 15 ml centrifuge tubes
15. 100 ml Glass beaker
16. Heating block for 1.5 ml eppendorff tubes
17. Coplin jars
18. Micropipettor, and cut and uncut tips
19. 1.5 ml eppendorff tubes
20. Cover glass (No.1, 24 × 50 mm)
21. Microscope Slide Tray (aluminum)

Preparation of Solutions:

1. PBS 10 × stock:
80 grams of NaCl
2 grams of KCl
2 grams of KH_2PO_4
11.5 grams of anhydrous Na_2HPO_4 or 29 grams of $Na_2HPO_4 \bullet 7H_2O$
32 grams Trizma Hydrochloride ($C_4H_{11}NO_3$, FW=157.6)
pH 7.4 (Phosphoric acid or HCl)
Autoclave and adjust volume to a liter and dispense in 10 ml aliquots.

2. Lysis and denaturing solution (can be prepared in advance and stored for up to one year):
1.25 M NaCl
1 mM tetra sodium EDTA
5 mM tris
0.01 % sodium lauroyl sarcosine

The following MUST be added FRESH to above solution:
0.2 % DMSO, final concentration
0.3 N NaOH, pH>13.5, added fresh in final concentration from a stock of 10 N NaOH

3. Neutralizing and DNA precipitating solution (can be prepared in advance and stored for up to one year):
Tris HCl 40 mM, pH 7.4
Spermine 2 mg/ml

The following MUST be added FRESH to above solution:
Ethanol 200 proof, diluted with above 1:1
(Ethanol final concentration 50%)
(Tris final concentration 20 mM)
(Spermine final concentration 1 mg/ml)

4. YOYO 1:
Stock solution of YOYO is 1 mM (Molecular Probe, Eugene, OR).
Working solution has 0.25 micromolar YOYO-1 in 2.5% DMSO and 0.5 % sucrose. Take 1 microliter of YOYO-1 and add to 4 ml of distlled water. Then add 200 microliters of DMSO sucrose solution mentioned below. Working staining solution can be dispensed in 500 microliters aliquots and stored at -20 degree C.
DMSO sucrose solution:
Fill a 100 ml bottle with 35 ml of distilled water
Dissolve 10 grams of sucrose in 35 ml of distilled water
Raise volume to 50 ml by adding distilled water
Add 50 ml of DMSO
This solution can be stored for a year.at room temperature:

<u>Preparation of agarose</u>:

1. 0.7% Agarose:
Suspend 70 mg of 3:1 high-resolution agarose (or Normal Melting agarose) in 9 ml distilled water in a 100 ml glass beaker and microwave until just boiling. This step should be repeated two more times for uniform heating. Add 1 ml of PBS 10× to the solution and mix. Microwave again to just boiling. Adjust the final volume up to 10 ml with dH_2O and microwave the solution again to just boiling. Dispense this agarose in 1.5 ml centrifuge tubes maintained at 55 degrees C in heat block.
[Note: Add agarose to water, not water to agarose powder]

2. 2% Agarose SFR:
Prepare 200 mg of SFR agarose in 10ml dH_2O with the same procedure as for 0.7% agarose. As 2% SFR agarose solution is viscous and has a tendency to stick to the bottom of the beaker, boiling on a hot plate in a beaker with a stirrer bar may be better way to prepare this agarose. Dispense the solution in 1.5 ml tubes and maintain at 55 degrees C in a heat block.
[**Note**: Both agarose solutions are made once and kept at 55 degrees C. They can be used for a week, but cannot be used after agarose solidifies. So it is better to make only as much agarose as can be used in on going experiments. However, if one can afford continuous heating of heat block, larger volumes can be prepared.]

<u>Preparation of MGE slides:</u>

1. Slide Precoating:
Place 50 microliters of 0.7% agarose on the MGE slide at one end and quickly spread with a pipette tip, so agarose solution spreads on the slide evenly.
Keep slides horizontal on a flat surface for drying.
[**Note:** Agarose preparation and slide precoating with agarose can be done days or months in advance of experiment]

2. Making the Microgel:
a) Mix 5 microliters of blood or sample cell suspension with 50 microliters of 0.7% agarose in an eppendorff tube and place on agarose precoated (dried) slide and cover with a cover glass. For agarose to solidify allow 5 min at room temperature or 1 min if slides are on ice.
b) Remove cover glass and place 200 microliters of SFR (2%) agarose on slide as final layer and quickly cover with a cover glass. For agarose to solidify allow 5 min at room temperature or 1 min on ice.
[Note: Use cut tips for making microgels on slides. The cover glass is held at an angle of 15 degrees over slide and quickly placed gently on top of agarose to spread the agarose. The SFR agarose is very viscous and gels very fast, hence care should be taken to place the cover glass quickly on agarose].

Lysing:

After removing cover glasses, immerse slides in a Coplin jar having 33 ml Lysing solution for 10 min at room temperature.

Neutralizing:
Take slides out of the lysing solution and immerse in a Coplin jar having 33 ml neutralizing solution for 30 min.
Transfer the slides into another Coplin jar with fresh neutralizing buffer for 30 min.

Drying:

After neutralizing, keep the slides on an aluminum slide tray and oven dry at 50 degrees C.
[**Note:** The dried slides can be kept for a longer period in dry conditions for later scoring. The slides may be stained and scored right after drying, or may be kept dry without staining. Once stained, the slides should be kept in a wet slide box and should be scored as early as possible.]

Staining:

Stain slides with the final working solution of YOYO-1 dye. To do this, apply 50 microliters of YOYO-1 in 5 small, equally spaced droplets over the clear window area and spread it gently by using pipette tip but without touching the slide and cover with cover glass for analysis. After staining, slides can be stored for a few days in a wet chamber (slide box having wet tissue paper) to retain the moisture.

Scoring:

The slides are analyzed using a florescent microscope with a blue filter (used for FITC excitation 490 nm, emission 510 nm and dichroic 500 nm) at magnification of 100 or 400×.
The number of total cells and the number of apoptotic cells in each field are counted and added together and the percentage of apoptotic cells is calculated. A minimum of 1000 cells per slide should be counted.