Comet Assay - Modified for Detection of Oxidized Bases Using the Repair Endonucleases Fpg, hOGG1 and Endonuclease III (Nth)

Overview

The Comet Assay (also called Single Cell Gel Electrophoresis) is used to assess DNA damage and repair in individual cells. The assay detects single and double-stranded DNA breaks by measuring the migration of DNA from individual nuclei following alkaline treatment. Migration of DNA out of the nucleus correlates closely to the number of single and double-stranded breaks present in the DNA.

Introduction

Acknowledgments and Explanations:
This protocol of Dr. Andrew Collins is from the Comet Assay Interest Group web site at https://cometassay.com/. It is a modification of a protocol developed by N.P. Singh (1). The protocol of Dr. Collins on the web site also discusses the use of lymphocytes for cells and/or UV treatment of these. The protocol below is limited to those steps tested at NEB.

Protocol

1. **Slide preparation:**
   Precoat clear microscope slides with agarose by dipping the slides into a staining jar of melted 1% **agarose** in H_20.
2. Drain off excess agarose and wipe the back of the slides clean.
3. Dry the dipped slides in a warm oven (37°C for several hours or until dry). Precoated slides can be stored until needed taking care to avoid high humidity conditions.

4. **Preparation of cells:**
   Treat cultured mouse fibroblast (NIH 3T3) or osteocyte cells with trypsin to detach the cells from the dish.
5. For each slide wash approximately 2 x 10^4 cells in 1 ml **PBS** in an Eppendorf tube by spinning at 200 x g, 3 minutes at 4°C.
6. After centrifugation remove as much of the supernatant as possible with a pipettor.

7. **Embedding cells in agarose:**
   Tap tube to disperse cells in the small amount of liquid remaining.
8. Add 70 µl of 1% low melting point **agarose** in PBS cooled to 37°C.
9. Pipette up and down to mix; then using the same tip, spot onto a precoated slide.
10. Cover with a 22 x 22 mm cover slip. Work rapidly as the agarose hardens quickly at room temperature.
11. Put slides at 4°C for 5 minutes.

12. **Treatment with Hydrogen Peroxide (H_2O_2):**
    Dilute **H_2O_2** to desired concentration in PBS. Remove cover slip. Spot 50 µl on agarose embedded cells.
13. Cover with a cover slip and put slides at 4°C for 5 minutes.

14. **Lysis:**
    Just before use add 1 ml Triton-X-100 to 100 ml of **lysis solution** chilled to 4°C.

15. Remove cover slips from slides and place slides in a staining jar containing lysis buffer.
16. Incubate 1 hour at 4°C. Use a separate staining jar for each treatment regimen.

17. **Enzyme treatment (either hOGG1 or Fpg):**
    Prepare 1X **enzyme reaction buffer**.
18. Reserve 1 ml for dilutions of the enzyme.
19. Wash slides in a staining jar with enzyme buffer 3 times, 5 minutes each wash.
20. Remove slides from last wash. Dab off excess liquid with a tissue.
21. Place 50 µl of the enzyme solution or buffer alone onto gel surface and cover with a 22 x 22 mm cover slip.
22. Put slides in a moist box (tupperware container with damp paper towels) and incubate at 37°C for 30 minutes. While the amount of enzyme required should be determined for each cell type and experiment we suggest first trying a $1{:}10^3$ to 10^4 dilution of Fpg and a $1{:}10^2$ or $1{:}10^3$ dilution for hOGG1.

23. **Alkaline treatment:**
    After incubation with the enzyme remove the cover slips and place slides on platform in an electrophoresis tank that contains the prechilled (4°C for at least one hour) **electrophoresis solution**. The buffer should just barely cover the slides.
24. Fill any gaps in the row with uncoated microscope slides.
25. Incubate 40 minutes at 4°C before beginning electrophoresis.

26. **Electrophoresis:**
    Run at 25V constant voltage for 30 minutes. Adjust the current to about 300 mA by raising or lowering the level of the electrophoresis solution.

27. **Neutralization:**
    Remove slides form electrophoresis apparatus and wash with 3 changes of **neutralization buffer** in staining jar for 5 minutes each at 4°C.

28. **Staining:**
    Spot 20 µl of a 1 µg/ml solution of **DAPI** in H_2O on the slide.
29. Cover with a cover slip. Place in a sealed container and keep in dark.

30. **Quantitation:**
    Using a fluorescence microscope estimate the tail length for 100 comets at random from each slide avoiding those comets on the edge of the gel as these are prone to artifacts.
31. Score each comet on a scale of 0 (no tail) to 4 (almost all DNA in the tail, insignificant head). Each slide can then be given an arbitrary score from 0 to 400.

    Notes: Controls which should be included for each experiment are: a no enzyme and no mutagen treatment; an enzyme treatment with no mutagen treatment; and a mutagen treatment with no enzyme treatment. Duplicate slides should be prepared for each condition.

32. **<u>Solutions</u>**

    Agarose:
    Electrophoresis grade, e.g. Invitrogen/LTI 5510UA
    LMP (low melting point) e.g. Invitrogen/LTI 5517US

    Phosphate-buffered saline (1X PBS):
    Dissolve 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 in 800 ml of distilled H_20. Adjust the pH to 7.4 with HCl. Add H_2O to 1 liter. Dispense the solution into aliquots and sterilize them by autoclaving for 20 minutes at 15 lb/square inch on liquid cycle. Store at room temperature.

    H_2O_2:
    Stock H_2O_2 is 8.8 M

Dilute 11.5 µl in 1 ml H_20 to give 0.1 M

Lysis Solution:
2.5 M NaCl
0.1 M EDTA Na_2
10 mM Tris-HCl, pH 10

Prepare 1 liter. Adjust pH to 10 with either solid NaOH or concentrated (10 M) NaOH solution. Add 1 ml Triton X-100 per 100 ml immediately before use.

Enzyme Reaction Buffer:
40 mM HEPES
0.1 M KCl
0.5 mM EDTA
0.2 mg/ml BSA
pH 8.0 with KOH
(can be made as a 10X stock, adjusted to pH 8.0 and frozen at -20°C)

Electrophoresis Solution:
0.3 M NaOH
1 mM EDTA

Neutralizing buffer:
0.4 M Tris
pH to 7.5 with concentrated HCl

DAPI:
1 mg/ml 4´6-diamidine-2-phenylindol dihydrochloride (DAPI) in distilled H_20. Store at -20°C.

References:

1. Singh, N., McCoy, M., Tice, R. and Schneider, L. (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. *Experimental Cell Research*, 175, 184–191.
2. Collins, A., Duthie, S. and Dobson, V. (1993). Direct enzymatic detection of endogenous oxidative base damage in human lymphocyte DNA. *Carcinogenesis*, 14, 1733–1735.
3. Collins, A., Dusinska, M., Gedik, C.,and Stetina, R. (1996). Oxidative damage to DNA: do we have a reliable biomarker? *Environmental Health Perspectives*, 104, 465–469.