Comet assay of cells from *N. furzeri* embryos

Slide Preparation

1. Melt 100 mL 1% Normal Melting Point Agarose in PBS in microwave
2. Pour agarose into a 50-ml tube and equilibrate to 50ºC in water bath
3. Dip SuperFrost Plus slides (clear with a frosted side, Fisher Scientific) into the agarose, up to the middle of the frosted edge
4. Wipe the backside of the slide clean with paper towel; lay on paper towel-covered trays to dry. Store in boxes at room temp.
5. Use slides from at least 1 to 3 weeks prior to the current experiment date

Harvest "0 time" or untreated cells:

Rinse cells with warm PBS (note: hard to detach cells may require rinsing with PBS containing EDTA), add 0.6 ml trypsin/EDTA, incubate at 37ºC for 2 min, add 0.6 ml cold medium, detach cells by pipetting.

Note: do not exceed trypsin treatment time. Alternatively, scrape cells with a rubber tip.

Transfer cells to Eppendorf tubes on ice, spin at 1550 rpm 3 min at 4°C. Remove supernatant, disperse pellet in 1 ml chilled PBS* (without Ca++ or Mg++). Spin again, and remove as much supernatant as possible using a pipettor. Repeat wash. Keep cells on ice or in the refrigerator until ready to layer on slides.

Cell number

Each gel should contain approximately 2-5x10^4 cells. Resuspend cells with 600 µl PBS*, or count and dilute to 10x10^5 cells/ml.

Embedding cells in agarose

Prepare 1.2% low melting point (LMP) agarose in PBS*, melt @ 70°C, keep @ 37°C. When ready, briefly warm the cells to 37°C, mix equal volumes of cells and agarose, keep at 37°C, spot 85 µl on a slide, cover with 24 x 60 mm coverslip (place so ~1mm overhangs at end), place at 4°C for 30 min to harden. Make at least 4 slides/sets.

Lysis

Add 0.5 ml Triton X-100 and 5 ml DMSO to 44.5 ml of pre-chilled lysis solution (4°C), making their final concentrations 1% and 10% individually. Bring slides to the cold room, remove coverslips by gently lifting from "overhangs", and place in a plastic box with a lid. Pour lysis and all other solutions gently along one side of the box, not on the slides to avoid dislodging the gels. Leave at 4°C 1 h overnight.

Alkaline treatment and Electrophoresis

1. Add 45 ml 10 N NaOH and 3 mL 0.5 M EDTA to 1.45 L dH_2O; keep at 4ºC until ready to use. Measure and ensure > pH13.

2. Electrophoresis must be run cold to avoid overheating the buffer. Place electrophoresis box in a plastic pan with ice/water mixture; make sure it's level; or set up buffer circulation through a flask in ice and a pump, or run it in the cold room.

3. Gently remove coverslips; put slides in electrophoresis box, with the frosted end facing the (red) anode

4. Add additional slides if necessary to cover the entire width of the box; can make several rows.

5. Add enough electrophoresis buffer to cover the slides.

6. Incubate for 40 minutes.

7. Turn on the power supply to 24V constant voltage.

8. Adjust the volume of electrophoresis buffer until 300 mA is reached (turn OFF power supply!)

9. Electrophorese for 30 minutes

10. Condition here is a common condition for most cell lines. If modification is required, pH, voltage, and electrophoresis time can be changed.

Neutralize

1. Wash slides with 0.4 M Tris pH 8 three times, 5 minutes each

Staining and Visualization

1. Prepare a solution with 1 ug/ml DAPI in 1x PBS; this can be stored in the dark and used repeatedly for > 1 year.
2. Immerse the slides in staining solution at room temperature in the dark for 15 min.
3. Gently dab with paper towel or tissue to dry all the moisture, or quickly dip slides very into 100% ethanol to dehydrate, and air-dry the slides.
4. Add 20 ul of Prolong Gold mounting medium. Cover with 24x60 mm coverslip and cure for 1 hour in the dark.
5. Water wash 2 times, 5 min each.
6. Cover with 24x60 mm coverslip and store at 4ºC until ready to view under microscope
7. To view, use 40x to 100x magnification with DAPI filter.

Others

1. To introduce DNA damage, treat cells with 200 uM H2O2 in PBS or HBSS for 5 min
2. With 3 lesions per million bases, ~75% DNA would fall into comet tails.

Lysis solution	**1 Liter**
2.5 M NaCl	146.1 g
0.1 M EDTA	37.2 g or 200 ml 0.5 M
10 mM Tris-HCL pH 8.0	10 ml 1 M

Prepare 1 liter. Set pH to 10 with either solid NaOH, or preferably concentrated (10 M) NaOH solution. *(Add 35ml of NaOH straight away to ensure that EDTA dissolves, and then add dropwise to pH 10.)*

Store in cold room.

Electrophoresis solution

0.3 M NaOH	30 ml 10 N
1 mM EDTA	3 ml 0.5 M

to 1.5 liter with water. pH should be 12. Chill before use.

Neutralizing buffer

0.4M Tris-HCl	24.25 g in 500 ml water

pH to 7.5 with conc. HCl (almost 12 ml).

Or 200 ml 1 M Tris.HCl pH 7.5 + 300 ml ddH_2O.

1.2 % LMP Agarose

LMP agarose UltraPure Invitrogen 15517-014

0.12 g in 10 ml PBS*; keep at 37°C.

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Adapted from the working protocol of

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