Research Center for
Pharmaceutical Nanotechnology
Tabriz University of Medical Sciences
RCPN

Comet Assay

PSR

The following “Comet assay” protocol provides an easy step-wise procedure for semi-quantitative detection of DNA damage

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Detailed procedure

Alkaline Comet Assay

The comet assay or single cell gel electrophoresis (SCGE) is a sensitive method for detecting strand breaks in the DNA of single cells with applications in genotoxicity testing and molecular epidemiology as well as fundamental research in DNA damage and repair. Although it is essentially a method for measuring DNA breaks.

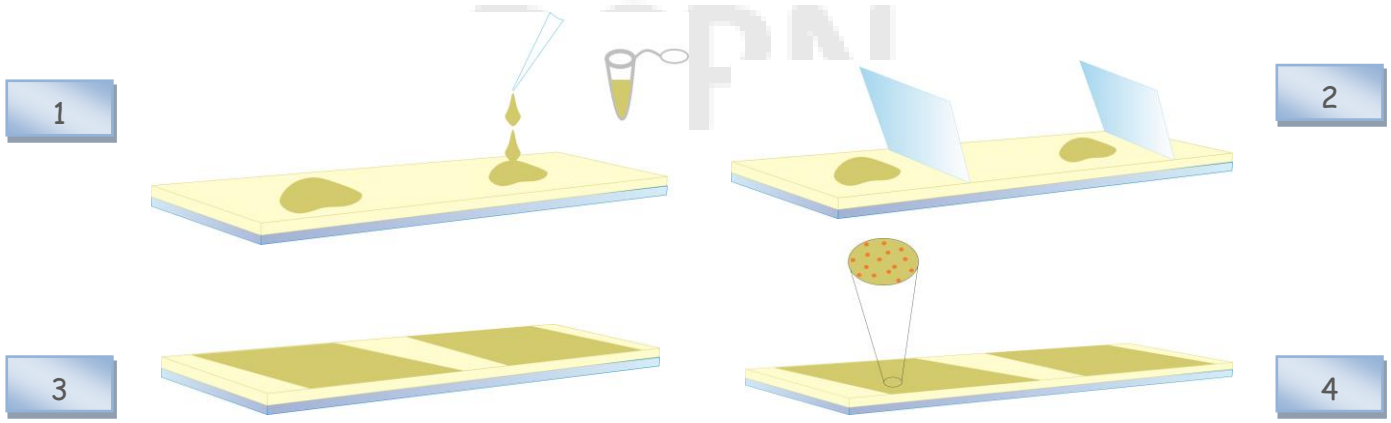
The assay attracts adherents by its simplicity, sensitivity, versatility, speed and economy. However the fact that it is so successful at demonstrating DNA damage is enough to justify its use in our research center.

1. **Precoating slides**

Prepare a layer of agarose by dipping a fully frosted microscope slide in to high melting point agarose (try to flat layer of agarose be formed on the slide surface). However after cleaning the other side of the slide, allow agarose embedded side of the slide to solidify at room temperature by following at 50 ºC for at least 30 min.

2. **Sample Loading**

Added second agarose layer with 75 µL of low melting point agarose with 10 µl cell suspension (10^6cell/mL) onto the first layer of agarose following by slipping a 24×24 coverslip on to second layer of agarose embedded cells gently. So by following slides in -20 ºC for at least 30 min (solidifying of the coverslip is very sensitive and important trick) gently remove the coverslip by tip fingernail.



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2.1. **Cell isolation / treatment**

a) Whole Blood:

To the pre-coated slide, add 75 µL of LMPA (0.5%; 51ºC) mixed with 5-15 µL heparinized whole blood. Either you can dilute blood with PBS as described in hematology procedure and add equal amount of 0.5% LMPA. (DMSO is added in the lysing solution to scavenge radicals generated by the iron released from hemoglobin when whole blood or animal tissues are used. Therefore it is not needed for other situations).

b) Cell cultures:

1. Monolayer Cultures:

Remove the media and add 0.005% Trypsin to the cells. Keep the cells at 370 C for 5 minutes to detach cells. (Very low concentration of Trypsin (0.005%) is used because higher concentrations increase DNA damage.) Then add equal amount of medium (with FBS) to quench Trypsin. Centrifuge the recent solvent (900g, 6 min) and remove the supernatant.

Mix ~10,000 cells in 10 µL or less volume per 75 µL LMPA and process accordingly

3. **Viability Assay**

1. Place 10 µL of at least 10^6 cells/mL in a microcentrifuge tube, and add 5 µL of trypan blue dye.
2. after two minutes place on a slide and put a coverslip.
3. Score 100 cells and record the number of viable cells (shiny) and dead cells (blue). (If the rate of viable cells become lower than 75%, repeat cell preparation AGAIN)

4. **Lysing step**

By dipping agarose embedded cells in 1% Triton X-100, 2.5 mM NaCl, 0.1 mM Na2EDTA, 10 mM Tris, pH 10 (Lysis solution) for at last 12 h (24 h is advised) allow to leaving out the whole structure of the cells (the step must carried out in dark room to avoiding more DNA damages).

5. **Naturalizing step**

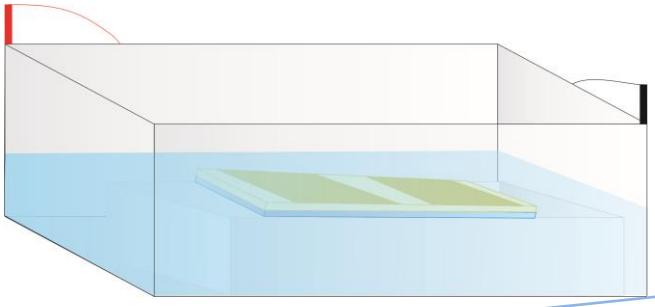
Wash the slides tree time in 0.4 M Tris-HCl, pH 7.5 (Naturalization buffer) at 4°C. (The step must carried out in dark room to avoiding more DNA damages)

6. **Unwinding step**

Immersed the slides into 0.3 M NaOH, 1 mM Na2EDTA, pH >13 (electrophoresis buffer) at 4°C for 20 min for unwinding without electricity. (The step must carried out in dark room to avoiding more DNA damages)

7. **Electrophoresis step**

Electrophores the slides at 18 V and 300 mA for 20 min in at least 4°C with the same solution that aforesaid. Follow washing the slides twice in 0.4 M Tris-HCl, pH 7.5 (Naturalization buffer) at 4°C.



8. **Staining and counting step**

Stain the slides with 25µLof 0.6 µM Etidium Bromide following by slipping a coverslip. From each cell line sample, select 100 randomly cells and analyze under fluorescent microscope by quantifying the DNA damage score with comet image analyzing system (such as CASP & Comet IV).

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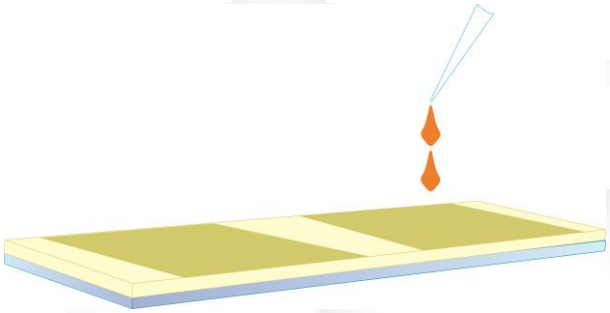

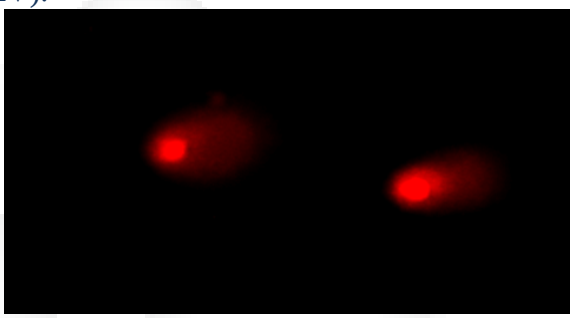

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Appendix

Solvents preparation

✓ Primary lysis buffer:

14.61 gr NaCl + 3.72 gr EDTA+ 1.2 gr Tris-Base+ 70 cc H20 +0.8 gr NaOH

Adjust pH to 10 using concentrated HCl or NaOH. Finally adjust the volume to 89 ml.

✓ Final lysis buffer:

Add 1% Triton X-100 or the other detergents such as CTAB or SDS to the required primary lysis buffer and then refrigerate for at least 30 min prior to the used

NOTE: In case of working with whole blood, use 10 % DMSO to the final lysis buffer. The purpose of the DMSO in lysis buffer is to scavenge radical generated by the iron released from hemoglobin.

✓ Electrophoresis buffer (300 mM NaOH/1mM EDTA)

Solvent A: 200 g NaOH+ 500 cc dH_2O (Store at room temperature)

Solvent B: 14.89 g EDTA + 200 cc dH_2O. Adjust pH to 10 (Store at room temperature)

✓ For 1X buffer:

30 ml solvent A+ 5 ml solvent B. Adjusts the volume to 1000 cc. prior to use, measure the pH of the buffer to ensure > 13

✓ Naturalization buffer:

48.5 gr Tris + 800 cc dH2O adjust pH to 7.5. Adjusts the volume to 1000 cc