Comet Assay Interest Group Website

January 2000 revision

THE COMET ASSAY
modified for detection of oxidised bases
with the use of bacterial repair endonucleases

1. Slide preparation

Use *either* fully frosted slides (which give good anchorage for agarose, but can only be used a few times before agarose layers begin to detach) *or* ordinary clear slides precoated with agarose by dipping in a (vertical) staining jar of melted 1% standard agarose in H_2O, draining off excess agarose, wiping the back clean and drying in a warm oven. The latter system has the advantage that, if slides are dried down after electrophoresis for storage and later examination, the comets (now very close to the glass surface) do not suffer from the high background fluorescence of the frosted surface.

Optional in the case of precoated slides, **normal** for frosted slides: In the middle of each slide, place 85-100 ?l of 1% standard agarose in PBS and, while still liquid, cover with a cover slip (18x18 or 22x22 mm). Place slides in fridge for at least 5 min for agarose to solidify. Push cover slip off first agarose layer with your thumb just before adding top layer (step 4 below).

2. Preparation of cells

Lymphocytes Take ~30 ?l blood from finger prick, or venous blood sample (+ anticoagulant). Add to 1 ml PBS (or 1ml RPMI medium + 10% FCS) in a 1.5 ml Eppendorf tube. Mix and leave on ice for 30 min. Then underlay with 100 ?l Histopaque 1077 (Sigma) or similar, using a pipettor. Spin at 200 x g, 3 min, 4°C. Retrieve lymphocytes in 100 ?l from just above boundary between PBS (RPMI) and Histopaque, using pipettor. Add to 1 ml PBS. Spin again. Remove as much supernatant as possible using pipettor.

Cultured (monolayer) cells Wash cells growing in dish with PBS, and add trypsin/EDTA; incubate until cells are rounding up, remove trypsin, add 1 ml of appropriate medium, detach cells by pipetting. Alternatively, use a silicon rubber scraper to remove cells. Transfer cells to Eppendorf tube, spin at 200 x g, 3 min, 4°C. Remove supernatant, disperse pellet in 1 ml PBS. Spin again, and remove as much supernatant as possible using pipettor.

Cell number When testing lymphocytes directly from whole blood, the 30 ?l sample suggested above should result in a reasonable density of comets (whether 1 or 2 gels

are made). Otherwise, if cultured cells, or lymphocytes from frozen storage are to be used, the density should be such that each gel contains approximately $2x10^4$ cells.

3. (Optional) Treatment with H_2O_2 ***[for UV treatment, see end of protocol]***

(NB. Do not use your own blood)

Stock H_2O_2 is 8.8 M
Dilute 11.5 ?l in 1 ml H_2O ? 0.1 M
Keep this as working stock for 1 week at most and then discard.
Dilute 10 ?l of 0.1 M solution in 1 ml PBS ? 1 mM
Further dilutions in PBS ? required concentrations for experiment.

e.g. 25µM = 975µl PBS + 25µl of 1mM solution.

Add to cells in tube or on dish. Leave on ice for 5 min. **Or**: after cells are embedded on agarose (step 4), add 50µl of required concentration of H_2O_2, cover with 22x22 coverslip, leave in cold room for 5 min.

4. Embedding cells in agarose

Tap tube to disperse cells in the small volume of medium remaining. Quickly add 140 ?l of 1% LMP agarose in PBS at 37°C and mix by tapping tube then sucking agarose up and down with pipette. Take 140 ?l of mixture (use same pipettor tip) and transfer as two roughly equal drops on each slide. Cover each with an 18x18 mm coverslip. Work quickly as the agarose sets quickly at room temperature! Leave slides in fridge for 5 min.

5. Lysis (1 h)

Add 1 ml Triton X-100 to 100 ml of lysis solution (4°C). Remove cover slips from slides and place in this solution in a (horizontal) staining jar. Leave at 4°C for 1 h. [If H_2O_2 has been used, any control slides must remain isolated, i.e. in separate vessels, during lysis; otherwise, strand breaks may occur in the control slides!]

6. Enzyme treatment (endonuclease III, formamidopyrimidine glycosylase)

Prepare 300 ml of enzyme reaction buffer. Put aside 1 ml for enzyme dilutions. Wash slides in 3 changes of this buffer (4°C) in staining jar, for 5 min each. Meanwhile, prepare dilutions of enzyme. The final dilution of the working solution will vary from batch to batch. Details for preparing the current batches (as at June 1998) are given under **Solutions, etc.** later in the protocol.

Note: The buffer in which the enzyme is prepared contains ?-mercaptoethanol to preserve the enzyme. However, inclusion of sulphydryl reagents in the reaction buffer would significantly increase background DNA breakage.

Remove slides from last wash, and dab off excess liquid with tissue. Place 50 µl of enzyme solution (or buffer alone, as control) onto gel, and cover with 22x22 mm cover slip. Put slides into moist box (prevents desiccation) and incubate at 37°C for 45 min (endo III) or 30 min (FPG).

7. Alkaline treatment (40 min)

Electrophoresis solution should be cooled before use, e.g. by pouring into the electrophoresis tank in the cold room an hour or so before it is needed. Gently place slides (minus cover slips) on platform in tank, immersed in solution, forming 1 or 2 complete rows (gaps filled with blank slides). Gels must be (just) covered. Leave 40 min.

8. Electrophoresis

30 min at 25 V (constant voltage setting). If there is too much electrolyte covering the slides, the current may be so high that it exceeds the maximum - so set this at a high level. If necessary, i.e. if 25 V is not reached, remove some solution.

9. Neutralisation

3 x 5 min washes with neutralising buffer in staining jar at 4°C.

10. Staining

Stain with 4'6-diamidine-2-phenylindol dihydrochloride (DAPI). Place 20 ?l of a 1 ?g/ml solution in distilled H_2O (stored at -20°C) onto each slide and cover with a 22x22 mm cover slip. Keep slides in a dark, moist chamber until they are viewed. They may be left overnight before viewing, either stained or unstained (however, if stained, some fluorescence is lost).
Alternative stains: Propidium iodide (2.5 ?g/ml), Hoechst 33258 (0.5 ?g/ml), or ethidium bromide (20 ?g/ml) can be used in place of DAPI for the visualisation of comet DNA.

11. Quantitation

Computer image analysis Several companies supply software which, linked to a closed circuit digital camera mounted on the microscope, automatically

analyses individual comet images. The programmes are designed to differentiate comet head from tail, and to measure a variety of parameters including tail length; % of total fluorescence in head and tail; and 'tail moment', calculated in different ways but essentially representing the product of tail length and relative tail intensity. *% DNA in tail* is linearly related to DNA break frequency up to about 80 % in tail, and this defines the useful range of the assay. *Tail length* tends to increase rapidly with dose at low levels of damage, but soon reaches its maximum. It is therefore the most sensitive parameter at near-background levels of damage. *Tail moment* is an attempt to combine the information of tail length and tail intensity, but suffers from lack of linearity.

Visual analysis It is possible to analyse comets quantitatively without image analysis software. The human eye readily discriminates comets representing different levels of damage, and we have developed a scheme for visual scoring based on 5 recognisable classes of comet, from class 0 (undamaged, no discernible tail) to class 4 (almost all DNA in tail, insignificant head) (see figure for examples). 100 comets are selected at random from each slide (avoid the edges of the gel, where anomalously high levels of damage are often seen). Each comet is given a value according to the class it is put into, so that an overall score can be derived for each gel, ranging from 0 to 400 arbitrary units. When slides are analysed in parallel by visual scoring and by computer image analysis, the match between results is excellent. With practice, visual scoring is very quick.

Calculation The control gels (no enzyme treatment) provide an estimate of the background of DNA strand breaks (SB). The enzyme-treated gels reveal strand breaks *and* oxidised bases (SB + OX). Assuming a linear dose response, whether working in *% DNA in tail* or in *arbitrary units*, subtraction of (SB) from (SB + OX) gives a measure of oxidised pyrimidines/altered purines.

Calibration Ionising radiation produces strand breaks in DNA with known efficiency. If the breaks introduced in cells by different doses of X-rays are detected with the comet assay, a standard curve can be drawn, with break frequency expressed as Gray-equivalents, or as breaks per unit length of DNA.

12. Storage and re-examination

Place slides in a warm oven until the gel has dried. Slides can then be stored at room temperature. For re-examination, stain as above. **Optionally**, add 100 ?l of 1% standard agarose on top of the original layers, cover with cover slip, leave to set, remove cover slip and stain as before.

SOLUTIONS, etc.

Prepare solutions from appropriate stocks, such as 0.5 M Na_2EDTA, 1 M Tris, 1 M KCl etc. Keep solutions at 4°C.

Lysis solution
2.5 M NaCl
0.1 M EDTA
10 mM Tris
Prepare 1 litre. 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.)* Add 1 ml Triton X-100 per 100 ml immediately before use.

Enzyme reaction buffer for endonuclease III and FPG
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 10 x stock, adjusted to pH 8.0 and frozen at -20°C)

Electrophoresis solution
0.3 M NaOH
1 mM EDTA

Neutralising buffer
0.4M Tris
pH to 7.5 with conc. HCl

Frosted microscope slides
supplied by: Richardson Supply Co., Robert Eliott Centre, Old Nichol Street, London E2 7HR (Fax 0171 7392137)
Cat. No. 267-096

Agarose
1. Electrophoresis grade, e.g. Gibco BRL 5510UA
2. LMP (low melting point), e.g. Gibco BRL 5517US

Enzymes
Endonuclease III (endo III) and formamidopyrimidine glycosylase (fpg) are isolated from bacteria containing over-producing plasmids. Because such a high proportion of protein is the enzyme, a crude extract is perfectly satisfactory; in our experience there is no non-specific nuclease activity. The enzyme extracts are best obtained from a laboratory producing them. On receipt, the enzyme (which should have been refrigerated in transit) should be dispensed into small

aliquots (say, 2 µl) and stored at -80°C. This minimises repeated freezing and thawing.

The final dilution of the working solution will vary from batch to batch.

Example (1); if **endo III** has a suggested dilution of 1000 x, dilute one 2 µl aliquot with buffer to 2ml, and store in suitable aliquots (e.g. 300 µl - enough for 6 gels) at -80°C.

Example (2): **fpg** is less stable than endo III, and repeated freezing/thawing must be minimised. If the suggested dilution is 3000 x, first dilute one 2 µl aliquot to 200 µl (100 x) and store 10 µl aliquots at -80°C. ***For this dilution, use buffer containing 10% glycerol.*** For use, dilute one 10 µl aliquot to 300 µl with buffer (no glycerol) and use at once; do not refreeze.

Centrifugation (our settings)

Jouan MR1822:
Radius: 74 mm Time/INT ? (1/0): 1 Time: 3 min 0 sec Temp: 4°C
Temp comp: 0°C Acc rate: 9 Brake: 0 RPM/RCF ? (1/0): 1 RCF: 200 g

TREATMENT OF CELLS

Treatment of cells with DNA-damaging agents, inhibitors, etc.:-
Cultured cells - treat in dishes before trypsinising (scraping) for suspension in agarose.
Lymphocytes - treat after the first spin, suspended in 1 ml of PBS or medium with serum.

Notes:

H_2O_2 treatment Stock H_2O_2 is 8.8 M
Dilute 11.5 ?l in 1 ml H_2O ? 0.1 M
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e.g. 25µM = 975µl PBS + 25µl of 1mM solution.
Add to cells in tube or on dish. Leave on ice for 5 min. **Or**: after cells are embedded on agarose (step 4), add 50µl of required concentration of H_2O_2, cover with 22x22 coverslip, leave in cold room for 5 min.

UV treatment, lymphocytes After PBS wash and spin, suspend cells in 1 ml of PBS and spin again (traces of Histopaque interfere with UV irradiation). Suspend pelleted cells in 100 ?l PBS, place on 35 mm dish and UV irradiate (without lid). Take 30 ?l

sample to mix with LMP agarose and proceed as in **3**. ***Or*** add 1 ml of medium ± aphidicolin *(30 ?l/ml of medium, from 2 mg/ml stock)* to each dish to follow repair during incubation at 37°C. At appropriate intervals spin cells and resuspend in LMP agarose etc.

For further details, contact:
Prof. Andrew Collins
Rowett Research Institute
Greenburn Road
Bucksburn
Aberdeen AB21 9SB
Scotland, UK

E-mail: a.collins@rri.sari.ac.uk