PROTOCOL FOR THE APPLICATION OF THE pH>13 ALKALINE SINGLE CELL GEL (SCG) ASSAY TO THE DETECTION OF DNA DAMAGE IN MAMMALIAN CELLS

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The alkaline (pH>13) SCG assay, developed by N.P. Singh[1], combines the simplicity of biochemical techniques for detecting DNA single strand breaks (frank strand breaks and incomplete excision repair sites), alkali-labile sites, and crosslinking with the single cell approach typical of cytogenetic assays. The advantages of the SCG technique include: (1) the collection of data at the level of the individual cell, allowing for more robust types of statistical analyses; (2) the need for small numbers of cells per sample (<10,000); (3) its sensitivity for detecting DNA damage; and (4) that virtually any eukaryote cell population is amenable to analysis. This protocol describes the methodology as used at ILS. Clearly, there are other methods to process samples, prepare gel slides, and analyze DNA damage; we would appreciate hearing about them. A Comet newsletter available to all interested individuals may be obtained from Kinetic Imaging Ltd. (South Harrington Building, Sefton Street, Liverpool, UK L3 4BQ, tel: 44(151)709 8633; FAX: 44(151)709-8633); E-MAIL: kinetic@btinternet.com.

The purpose of this protocol is to provide instruction on the application of the alkaline SCG assay to the detection of DNA damage in eukaryote cells obtained from *in vitro* and *in vivo* studies. For a review, see R.R. Tice (1995) The single cell gel/comet assay: a microgel electrophoretic technique for the detection of DNA damage and repair in individual cells. In: Environmental Mutagenesis (D.H. Phillips and S. Venitt, eds.), Bios Scientific Publishers, Oxford, 315-339.

[1]Singh, N.P., McCoy, M.T., Tice, R.R. and Schneider, E.L. (1998) A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell Res. 175: 184-191.

I. Preparation of Reagents

Materials:	Supplier (Catalogue Number):
Dimethylsulfoxide (DMSO)	- Fisher (D128-500)
Disodium EDTA	- Sigma (E-5134)
Ethidium Bromide	- Sigma (E-8751)
Hanks Balanced Salt Solution (HBSS) (Ca^{++}, Mg^{++} free)	- GIBCO (1804873)
Phosphate Buffered Saline (PBS) (Ca^{++}, Mg^{++} free)	- GIBCO (450-1300EC)
Sodium Chloride (NaCl)	- Sigma (S-9625)
Sodium Hydroxide (NaOH)	- Sigma (S-5881)
SYBR™ Green I	- Molecular Probes, Inc., P.O. Box 22010, Eugene, OR, 503-465-8353
Triton X-100	- Sigma (x-100)
Trizma Base	- Sigma (T-8524)

Procedure:

1. Mincing Solution: HBSS (Ca^{++}, Mg^{++} free) with 20 mM EDTA and 10% DMSO: To 400 mL 1x HBSS (Ca^{++}, Mg^{++} free), add 3.72 g EDTA and 50 mL DMSO, adjust pH to 7.0 - 7.5., q.s. to 500 mL, store at room temperature.

2. PBS (Ca^{++}, Mg^{++} free): Dulbecco's PBS - 1 L packet: add 990 mL dH_2O, adjust pH to 7.4, q.s. to 1000 mL, store at room temperature.

3. Lysing Solution: Ingredients per 1000 mL: 2.5 M NaCl 146.1 gm
   100 mM EDTA- 37.2 gm
   10 mM Trizma base 1.2 gm

   Add ingredients to about 700 mL dH_2O, and begin stirring the mixture. Add ~8 gm NaOH and allow the mixture to dissolve (about 20 min). Adjust the pH to 10.0 using concentrated HCl or NaOH. q.s. to 890 mL with dH_2O (the Triton X-100 and DMSO will increase the volume to the correct amount), store at room temperature.

   Final lysing solution: add fresh 1% Triton X-100 and 10% DMSO, and then refrigerate for at least 30 minutes prior to slide addition.

   NOTE: The purpose of the DMSO in the lysing solution is to scavenge radicals generated by the iron released from hemoglobin when blood or animal tissues are used. It is not needed for other situations or where the slides will be kept in lysing for a brief time only. Sodium lauryl sarcosinate, which we used earlier, is not included in this version, but may be needed for some cell types.

4. Electrophoresis Buffer (300 mM NaOH / 1 mM EDTA):

   Prepare from stock solutions:
   1. 10 N NaOH (200 g/500 mL dH_2O)
   2. 200 mM EDTA (14.89 g/200 mL dH_2O, pH 10)

   store both at room temperature. We prepare the NaOH and EDTA stock solutions every ~2 weeks.

   For 1X Buffer (made fresh before each electrophoresis run): per liter, add 30 mL NaOH and 5.0 mL EDTA, q.s. to 1000 mL, mix well. The total volume depends on the gel box capacity. Prior to use, measure the pH of the buffer to ensure >13.

   NOTE: Use this buffer at pH 12.1 to selectively assess for strand breakage.

5. Neutralization Buffer: 0.4 M Tris - 48.5 gm added to ~800 mL dH_2O, adjust pH to 7.5 with concentrated (>10 M) HCl: q.s. to 1000 mL with dH_2O, store at room temperature.

6. Staining Solution: Ethidium Bromide (10X Stock - 20 µg/mL): add 10 mg in 50 mL dH_2O, store at room temperature. For 1X stock - mix 1 mL with 9 mL dH_2O.

   SYBR™ Green: Add 1 µL of provided stock solution to 10 mL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) to prepare a 1:10,000 dilution. Make fresh prior to use. Staining solution is stable for several hours at room temperature and for 1-2 days when refrigerated. pH is critical for stability.

CAUTION: Handle dyes appropriately.

NOTE: SYBR™ Green I offers a greatly increased signal to noise ratio. However, as the stain fades rapidly upon exposure to light, the DNA must be stained just prior to analysis. Other dyes used include acridine orange, propidium iodide, Hoechst 33258, etc.

II. Preparation of Slides for the Single Cell Gel Assay

Materials:	Supplier (Catalogue Number):
Coplin jars (opaque)	- Fisher (08-815-10)
Coverslips (No. 1, 24 x 50 mm)	- Fisher (12-545F)
Frozen Ice Packs	
Low Melting Point Agarose (LMPA)	- Fisher (BP165-25)
Microcentrifuge Tubes (for freezing samples)	- Krackler (383-MCT-150A)
Micropipettor and Tips	- Rainin
Microscope Slides, Conventional	- Fisher (12-550-34)
Microscope Slide Tray (aluminum)	- Shandon Lipshaw (203)
Microscope Slide Tray (plastic covered)	- Shandon Lipshaw (1760)
Normal Melting Agarose (NMA)	- Fisher (BP1365-100)
PBS (Ca^{++}, Mg^{++} free)	- GIBCO (21600-010)
Scintillation vials	- Fisher (03-337-4)
ShurMark Marker for labeling slides	- Fisher (15-183-10)

Procedure:

1. Prepare 0.5% LMPA (250 mg per 50 mL) and 1.0% NMA (500 mg per 50 mL) in PBS. Microwave or heat until near boiling and the agarose dissolves. For LMPA, aliquot 5 mL samples into scintillation vials (or other suitable containers) and refrigerate until needed. When needed, briefly melt agarose in microwave or by another appropriate method. Place LMPA vial in a 37ºC water bath to cool and stabilize the temperature.

2. While NMA agarose is hot, dip conventional slides up to one-half the frosted area and gently remove*. Wipe underside of slide to remove agarose and lay the slide in a tray on a flat surface to dry. The slides may be air dried or warmed for quicker drying. Store the slides at room temperature until needed; avoid high humidity conditions. We generally prepare slides the day before use.

   NOTE: Slides should be labeled prior to dipping.

*Procedure based on information provided in M. Klaude, S. Eriksson, J. Nygren, and G. Ahnstrom (1996) The comet assay: mechanisms and technical considerations. Mutation Res. 363: 89-96.

3. To the coated slide, add 75 Lµ of LMPA (37ºC) mixed with ~10,000 cells in ~5-10 µL (do not use more than 10 µL). Replace coverslip and place the slide on a slide tray resting on the ice packs until the agarose layer hardens (~3 to 5 minutes). Using ~10,000 cells in an area 24 by 50 mm results in ~1 cell per microscope field at 200-250 x magnification.

4. Gently slide off coverslip and add a third agarose layer (75 µL LMPA) to the slide. Replace coverslip and return to the slide tray until the agarose layer hardens (~3 to 5 minutes).

5. Remove coverslip and slowly lower slide into cold, freshly made Lysing Solution. Protect from light and refrigerate for a minimum of 1 hour. In our experience, slides may be stored for at least 4 weeks in cold Lysing Solution without affecting the results.

NOTE: The amounts indicated are based on using 24 x 50 mm coverslips. Proportional volumes can be used for coverslips differing in size. If the gels are not sticking to the slides properly, avoiding humidity and/or increasing the concentration of NMA agarose in the lower layer to 1.5% should eliminate the problem. Steps 3 to 5 should be performed under dim yellow lights to prevent DNA damage.

III. Cell Isolation

1. Whole Blood: Per gel slide, mix ~5 µL whole blood with 75 µL LMPA and process accordingly.

NOTE: A small volume of blood can be added to 1 mL of media and stored cold (refrigerated or on ice) for an extended period of time until processed. In this case, the cells must be pelleted by centrifugation and care taken to remove as much supernatant as possible before adding the 75 µL LMPA per 5 µL of blood. In cases where protracted sample storage is necessary, this sample can be flash-frozen in liquid nitrogen and then stored at –70?C until processed. Flash-freezing optimizes DNA integrity but not necessarily cell survival.

2. Isolated Lymphocytes: Micro technique: Mix 20 µL whole blood with 1 mL RPMI 1640 in a microcentrifuge tube, add 100 µL Ficoll below the blood/media mixture. Spin for 3 min at 2000 x g. Remove 100 µL of bottom of the media/top of Ficoll layer, add to 1 mL media and mix, spin for 3 min to pellet lymphocytes. Pour off supernatant, resuspend pellet in 75 µL LMPA, and process accordingly.

3. Bone Marrow: Perfuse a femur (mouse) with one mL of cold mincing solution (HBSS with 20 mM EDTA, 10% DMSO) into a microcentrifuge tube. Remove and mix 5 µL per 75 µL LMPA, and process accordingly.

4. Solid Organs: Place a small piece of an organ in 1 mL of cold HBSS containing 20 mM EDTA/10% DMSO. Mince into fine pieces, let settle, remove and mix 5 - 10 µL of the cell suspension per 75 µL LMPA, and process accordingly.

NOTE: For blood rich organs (e.g., liver), mince into large pieces, let settle, aspirate mincing solution, add fresh mincing solution, mince into finer pieces, remove and mix 5 µL of the cell suspension with 75 µL LMPA, and process accordingly. The purpose of the DMSO is to prevent lipid peroxidation associated with the processing of some tissue. The volume of the cell suspension to mix with 75 µL of LMPA must be 10 µL or less, while the optimal cell number is ~10,000 cells per slide. Adjust the volume of mincing solution as needed. In cases where protracted sample storage is necessary, the minced sample can be flash-frozen in liquid nitrogen and then stored at –70?C until processed. Flash-freezing optimizes DNA integrity but not necessarily cell survival.

5. Monolayer Cultures: Remove the media and replace with mincing solution, scrape off cells into the mincing solution using a teflon scrapper to yield approximately $1x10^6$ cells/mL. Remove and mix 5 - 10 µL of the cell suspension per 75 µL LMPA and process accordingly.

NOTE: Trypsin can be used but excessive amounts/times increases DNA damage.

6. Suspension Cultures: Mix ~10,000 cells in 10 ?L or less volume per 75 ?L LMPA and process accordingly.

7. Viability Assessment: Viability assessment is often critical for interpreting SCG data. While several methods are available, we routinely use a technique developed by G.H.S. Strauss (Non-random cell killing in cryopreservation: Implications for performance of the battery of leukocyte tests (BLT) I. Toxic and immunotoxic effects, Mutation Res. 252: 1-15, 1991).

Materials:

Solution A: ethidium bromide (EB; Sigma #E8751). For the stock solution, dissolve 50 mg EB in 1.0 mL 100%-EtOH, and add 100 µL to 4.9 mL PBS. For the working solution, add 250 µL stock solution to 9.75 mL PBS (final conc. = 0.025 µg/µL), and protect from light.

Solution B: 5-6 carboxyfluorescein diacetate (CFDA; Sigma #C-8166). For the stock solution, dissolve 3 mg CFDA in 1.0 mL acetone. For the working solution, add 420 µL stock solution to 9.58 mL PBS (final conc. = 0.125 µg/µL), and protect from light.

Solution C: Working solution: Combine A and B in a ratio of 1:1. Can be refrigerated for up to 6 months. Protect from light.

Procedure:

1. Place 40 µL of at least 10^6 cells/mL in a microcentrifuge tube, and add 10 µL of working solution of dual stain (5-6 carboxyfluorescein diacetate:ethidium bromide; solution C).

2. Let stand for 3-5 min at 37ºC, and then remove excess stain by washing cells with 1 mL of wash solution (tissue culture media, PBS, etc).

3. Pellet cell sample, and pour off supernatant.

4. Repeat wash. The cells may be stored by placing pelleted cells in PBS at 4ºC until ready to score.

5. Pellet cells, and pour off supernatant. Mix cells in remaining drop of fluid (about 10 µL).

6. Place drop with cells on microscope slide, add coverslip (the smaller in size, the better).

7. Observe cells with fluorescent microscope (FITC or acridine orange filter combination).

8. Score 100 cells for the number of viable cells (green fluorescence in cytoplasm), the number of compromised cells (green fluorescence in cytoplasm, red fluorescence in nucleus), and the number of dead cells (red fluorescence in nucleus).

   NOTE: In this staining technique, a live cell will metabolize 5-6 carboxyfluorescein diacetate to a fluorescence compound, turning the cytoplasm green. The ethidium bromide will stain the nucleus of any cell with a damaged cell membrane. Excessive exposure to UV light will kill cells, resulting in uptake of ethidium bromide (red stain), if available. In compromised cells, the intensity of cytoplasmic stain will vary greatly, depending on how metabolically competent is the cell.

IV. Electrophoresis of Microgel Slides

Materials:	Supplier (Catalogue Number):
Coverslips (No. 1, 24 x 50 mm)	- Fisher (12-545F)
Micropipettor and Tips	- Rainin
Ethanol or methanol	
formalin	

Procedure:

NOTE: The procedure described is for electrophoresis under pH>13 alkaline conditions. Electrophoresis can also be conducted at pH 12.1 or under neutral conditions. Also, slides can be removed from lysis to treat with repair enzymes specific for different classes of DNA damage or with proteinase K to remove any residual protein (or to distinguish between DNA-DNA and DNA-protein crosslinking , or can be used under nonelectrophoretic conditions to detect apoptotic/necrotic cells.

1. After at least 1 hour at ~4ºC, gently remove slides from the Lysing Solution. Rinse slides carefully in Tris (e.g., 3 x 5 min) to remove detergents and salts. Place slides side by side on the horizontal gel box near one end, sliding them as close together as possible.

2. Fill the buffer reservoirs with freshly made pH>13 Electrophoresis Buffer until the liquid level completely covers the slides (avoid bubbles over the agarose).

3. Let slides sit in the alkaline buffer for 20 to 60 minutes to allow for unwinding of the DNA and the expression of alkali-labile damage.

   NOTE: The longer the exposure to alkali, the greater the expression of alkali-labile damage.

4. Turn on power supply to 25 volts (~0.74 V/cm) and adjust the current to 300 milliamperes by raising or lowering the buffer level. Depending on the purpose of the study and on the extent of migration in control samples, electrophorese the slides for 10 to 40 minutes.

   NOTE: The goal is to obtain migration among the control cells without it being excessive. The optimal electrophoresis duration differs for different cell types. If crosslinking is one of the endpoints being assessed then having controls with about 25% migrated DNA is useful. A lower voltage, amperage, and a longer electrophoresis time may allow for increased sensitivity. Different gel boxes will require different voltage settings to correct for the distance between the anode and the cathode.

5. Turn off the power. Gently lift the slides from the buffer and place on a drain tray. Dropwise coat the slides with Neutralization Buffer, let sit for at least 5 minutes. Drain slides and repeat two more times.

   NOTE: Slides may be stained with 100 µL 1X Ethidium Bromide or SYBR™ Green I stain and scored immediately or dried as in step 6.

6. Drain slides, expose them (e.g., 5 min) to cold 100% ethanol or cold 100% methanol, and allow to dry. Store in dry area.

NOTE: we have considered the possibility that treating slides with formalin (e.g., 10 min) in lieu of alcohol or after alcohol treatment will increase the stability of the gels during storage, and have implemented this procedure. We have no data yet to support this step but see no reason it would not be helpful..

7. When convenient, flood the slide with stain (~300-500 µL) before placing coverslip to ensure even hydration and staining and cover with a fresh coverslip. Before viewing slides, blot away excess liquid on the back and edges. After scoring, remove coverslip, rinse in 100% alcohol to remove stain, let dry, and store.

NOTE: Perform steps 1 through 4 under yellow light; the premise is that usual lighting will cause DNA damage but have no data to support this concern.

V. Evaluation of DNA Damage

1. For visualization of DNA damage, observations are made of fluorochrome-stained DNA using a 20-25x objective on a fluorescent microscope.

NOTE: Depending on the size of the cells being scored, other objectives can be used (i.e., 16x, 40x).

2. Although any image analysis system may be suitable for the quantitation of SCG data, we use a Komet analysis system 4.0 developed by Kinetic Imaging, LTD (Liverpool, UK) linked to a CCD camera to quantitate the length of DNA migration and the percentage of migrated DNA. To distinguishing between populations of cell differing in size (e.g., parenchymal versus nonparenchymal cells), we also measure nuclear diameter. Finally, the program calculates tail moment. Generally, 50 to 100 randomly selected cells are analyzed per sample.

3. Statistical analysis for *in vitro* data is based on multiple cultures, and for *in vivo* data on a minimum of 4 animals per group (suggest 5 to 8 as being more appropriate). In addition, we have analyzed the number of cells which exhibit values greater than the 95 or 99% confidence limits for the distribution of control data (i.e., the frequency of damaged versus undamaged cells).

NOTE: Although we use an image analysis system to collect the data, other approaches can be used. For example, the frequency of round cells, cells with short tails, cells with medium long tails, and cells with long tails can be counted among 100 cells and analyzed. To evaluate if the assay is functioning correctly, a control and treated (ex. 0.1.0 Gy x-ray), cryopreserved population of cells (e.g., blood lymphocytes) can be used. The treated cell population can be stored as 1 mL aliquots (at a density of 100,000 cells per mL) into microcentrifuge tubes. Whenever cells are being electrophoresed, one of these "internal control" cell samples can be processed along with the study population, and analyzed for DNA damage. The SCG data resulting from this population can be used to evaluate interrun variability.

VI. A Model Experiment: Human Leukocytes and Hydrogen Peroxide (H_2O_2)

This experiment consists of a comparison of basal and chemically-induced levels of DNA damage among human leukocytes. For the former, DNA migration among cells electrophoresed for either 20 or 40 minutes will be compared. For the latter, leukocytes will be exposed to H_2O_2, an excellent inducer of DNA damage. Because of its ease of use, H_2O_2 makes for a good positive control while the SCG assay is being developed.

1. Transfer 20 µL of whole blood to 1 mL of HBSS or 1 mL of HBSS containing 200 µM H_2O_2 in a microcentrifuge tube in duplicate per treatment group.

2. Place samples in a refrigerator or in a 37ºC incubator for 60 minutes.

3. Prepare 2 lysing jars (50 mL coplin jar containing 40 mL lysing solution) and place in the refrigerator.

4. Label four slides for each tube, dip slides in regular agarose, and allow to dry.

5. At the end of the treatment period, pellet the cells, remove supernatant, resuspend cells in HBSS, and remove 500 µL for viability assessment.

6. Pellet the cells for SCG analysis.

7. After removing as much of the supernatant as possible, resuspend each pellet in 300 µL 0.5% LMPA and layer 75 µL onto each of four labeled microscope slides. Place the slides on the cold slide tray.

8. After allowing the agarose to solidify, add the final layer of 0.5% LMPA (75 µL). Replace the slides on the cold slide tray.

9. After this layer solidifies, remove coverslips and place slides back to back vertically in a 50 mL coplin jar containing 40 mL of cold Lysing Solution. Place the coplin jars in the refrigerator for a minimum of 1 hour.

10. Transfer 2 slides from each tube within each treatment group (8 slides total) to the gel box, expose the DNA to alkali for 20 min, and then electrophorese for 20 minutes.

11. Neutralize with Tris buffer and stain with ethidium bromide or another appropriate dye (wear disposable gloves for this step).

12. Score 50 cells per duplicate slide for DNA migration using image analysis, or score 100 cells per slide by eye, categorizing cells as undamaged (i.e., no migration), short migration, medium migration, long migration, and complete migration (i.e., no nucleus remaining).

13. Repeat steps 9 through 11 using 40 minutes of electrophoresis.

14. Compare the amount of migration per cell, the number of cells with increased migration, the extent of migration among damaged cells, and viability.