PROTOCOL FOR THE SINGLE CELL GEL ELECTROPHORESIS / COMET ASSAY FOR RAPID GENOTOXICITY ASSESSMENT

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The single cell gel electrophoresis (SCGE)/ comet 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 SCGE 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 eukaryotic cell population is amenable to analysis.

A working protocol that can be used to detect DNA damage is given below:

I. Preparation of Reagents

Materials:	Supplier (Catalogue Number):
Dimethylsulfoxide (DMSO)	- Qualigens (CPW59)
Disodium EDTA	- HiMedia (RM1370)
Ethidium Bromide	- Sigma (E-8751)
Histopaque	- Sigma (1077-1)
Phosphate Buffered Saline (PBS) (Ca^{++}, Mg^{++} free)	- HiMedia (TS1006)
Sodium Chloride (NaCl)	- Ranbaxy Rankem (S0160)
Sodium Hydroxide (NaOH)	- BDH-Merck (89021)
Triton X-100	- HiMedia (RM 845)
Trizma Base	- Spectrochem (042061)

Procedure:

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

2. 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.

3. 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.

4. 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.

5. Staining Solution: Ethidium Bromide (EtBr; 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.

CAUTION: Handle EtBr with adequate precaution as it is known carcinogen.

II. Preparation of Slides for the SCGE/Comet Assay

Materials:	Supplier (Catalogue Number):
Normal Melting Agarose (NMA)	- HiMedia (RM273)
Low Melting Point Agarose (LMPA)	- Sigma (A9414)
Methanol	- Qualigens
Coverslips (No. 1, 24 x 60 mm)	- Blue Star
Microcentrifuge Tubes	- Tarsons (500010)
Micropipettor and Tips	- Tarsons
Microscope Slides, Conventional / Micro gel electrophoresis (MGE) slides	- Blue Label or Es Em Inc (NS 0001)
Coplin jars (opaque)	- Tarsons (480000)
Horizontal Gel Electrophoresis Apparatus	-GIBCO BRL, Life Technologies

Electrophoresis Power Supply - Techno Source
Microscope Slide Tray (aluminum)

A. Preparation of base slides

1. Prepare 1% (500 mg per 50ml PBS) and 0.5% LMPA (250 mg per 50 ml PBS) and 1.0% NMA (500 mg per 50 ml in Milli Q water). 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 dry/water bath to cool and stabilize the temperature.

2. Dip the slides in methanol and burn them over a blue flame to remove the machine oil and dust.

3. While NMA agarose is hot, dip conventional slides up to one-third the frosted area and gently remove (2). 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 at 50^0C 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 before storage.

B. Cell isolation / treatment

a) Whole Blood:

1. To the coated slide, add 75 µL of LMPA (0.5%; 37ºC) mixed with ~10,000 lymphocytes in ~5-10 µL (do not use more than 10 µL) *OR DILUTE BLOOD WITH PBS AND ADD EQUAL VOLUMES OF DILUTED BLOOD AND 1% LMPA.* Place coverslip and put the slide on a slide tray resting on ice packs until the agarose layer hardens (~5 to 10 minutes).
2. Gently slide off coverslip and add a third agarose layer (80 µL LMPA) to the slide. Replace coverslip and return to the slide tray until the agarose layer hardens (~5 to 10 minutes).

b) Isolated Lymphocytes:

Micro technique: Mix 20 µL whole blood with 1 mL RPMI 1640 in a microcentrifuge tube, add 100 µL Ficoll histopaque 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 as above.

c) Separation of Lymphocytes and treatment *in vitro*:

1. 1 ml of blood is taken from a healthy donor by venipuncture. Lymphocytes are separated from it using Histopaque-1077. Briefly, blood is diluted 1:1 with PBS or RPMI (without FBS) and layered over 600 ul Histopaque and centrifuged at 800 X g for 20 minutes. The 'buffy' coat is aspirated into 3-5 ml of PBS/ RPMI and centrifuged at 250 X g for 10 minutes to pellet the lymphocytes. The pellet is resuspended in ~1 ml of IRPMI and counted over a Haemocytometer. Nearly 2 X 10^4 cells per 100 µL of medium are taken for each dose of the test material.
2. One ml of each dose is made in medium (without FBS) and lymphocytes added to it. The eppendorff is inverted to mix the cells and test material.
3. The ependorff-tubes are properly wiped with alcohol and kept in the incubator at 37^0C for 3 hours.

4. After the treatment, the cells are centrifuged at 3000 rpm for 5 minutes to pellet the lymphocytes. The test substance is aspirated and discarded. The pellet is then resuspended in 100 ml of PBS & 10 ml removed for trypan blue viability test.
5. 100 ml of 1% LMPA is added & 80 ml of the suspension layered onto the base slides, and a coverslip is placed on it. Put the slides on ice packs until Agarose layer hardens (~5 to 10 min).
6. Gently slide off coverslip and add a third agarose layer (90 µL LMPA) to the slide. Replace coverslip and return to the slide tray until the agarose layer hardens (~5 to 10 minutes).
7. Remove coverslip and slowly lower slide into cold, freshly made Lysing Solution. Protect from light and refrigerate for a minimum of 2 hours.

d) Bone Marrow:

Perfuse a femur (mouse) with one mL of cold mincing solution (HBSS with 20 mM EDTA, 10% DMSO) into a microcentrifuge tube. Mix 5 µl per 75 µl LMPA, and process accordingly.

e) Solid Organs/tissues:

Place a small piece of an organ/ tissue in 1 mL cold HBSS containing 20 mM EDTA/10% DMSO. Mince into fine pieces, let settle, remove and mix 5 - 10 µl with 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.

f) Cell cultures:

1. Monolayer Cultures:
Remove the media and replace with mincing solution, scrape off cells into the mincing solution using a teflon scraper to yield approximately 1x10 cells/mL. Remove and mix 5 - 10 µl of the cell suspension per 75 µl LMPA and process accordingly. ***OR***
Remove the media and add 0.005% Trypsin to the cells. Keep the cells at 37^0 C for 5 minutes to detach cells. (Very low concentration of Trypsin (0.005%) is used because higher concentrations increase DNA damage.)
Add equal amount of medium (with FBS) to quench Trypsin.
Mix ~10,000 cells in 10 µl or less volume per 75 µl LMPA and process accordingly

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

NOTE: The amounts indicated are based on using No.1, 24 x 60mm 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 4 to 6 should be performed under dim yellow lights to prevent DNA damage.

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. Let stand for at least two minutes and then place on a slide and put a coverslip.
3. Score 100 cells and record the number of viable cells (shiny) and dead cells (blue).

III. Electrophoresis of Microgel Slides

The procedure described is for electrophoresis under pH>13 alkaline conditions.

1. After at least 2hour at ~4ºC, gently remove slides from the Lysing Solution. 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 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 24 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 30 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. Drop wise coat the slides with Neutralization Buffer, let sit for at least 5 minutes. Drain slides and repeat two more times.

6. Slides may be stained with 80µL 1X Ethidium Bromide, leave for 5 min and then dipped in chilled distilled water to remove excess stain. The coverslip is then placed over it and the slides are scored immediately or dried before staining as in step 7.

7. Drain slides, keep them for 20 min in cold 100% ethanol or cold 100% methanol for dehydration. Air dry the slides and place them in an oven at 50^0C for 30 min. Store in a dry area.

8. When convenient, rehydrate the slides with chilled distilled water for 30 min and stain with EtBr as in step 6 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 for archival purposes if needed.

   NOTE: Perform steps 1- 4 under yellow / dimmed light. This is to prevent any DNA damage that may arise from fluorescent white light.

IV. Evaluation of DNA Damage

1. For visualization of DNA damage, observations are made of EtBr-stained DNA using a 40x objective on a fluorescent microscope.

2. Although any image analysis system may be suitable for the quantitation of SCGE data, we use a Komet 5 image analysis software developed by Kinetic Imaging, Ltd. (Liverpool, UK) linked to a CCD camera to assess the quantitative and qualitative extent of DNA damage in the cells by measuring the length of DNA migration and the percentage of migrated DNA. Finally, the program calculates tail moment. Generally, 50 to 100 randomly selected cells are analyzed per sample.

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

For further reading, one is referred to the following literature:

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Addresses of suppliers:

1. Sigma Chemical Co.
Survey # 31/1, Sitharamapalay, Mahadevapura, P.O. Bangalore-560048. Tel: (080) 8524222/8524149/8524216. FAX: (080) 8524214
E-mail: sigma@blr.vsnl.net.in; sigma.aldrich@gnblr-sigma.global.net.in

Sector B-5/6, Flat # 4082, Vasant Kunj, New Delhi – 110 070. Tel: (011) 26899826 / 26897830 FAX: (011) 2699827. E-mail: sigma@del2.vsnl.net.in

2. Merck
E. Merck (India) Limited, Shiv Sagar Estate 'A', Dr. Anne Besant Road, Worli, Mumbai – 400018. Tel: (022) 4964855/4962612. FAX: (022) 4950354/4950307/4954590.
E-mail: lb.mktg@emil.sprintsmx.ems.vsnl.net.in

3. Tarsons Products Pvt. Ltd.
856 Marshall House, 33/1 Netaji Subhash Road, Kolkatta – 700001. Tel: (033) 2204022/209025/2211013. FAX: (033) 2211014. Website: www.tarsonsproducts.com.
E-mail: tarsons@satyam.net.in.

4. Blue Label Scientifics Pvt. Ltd.
F/3, Shree Laxmi Woollen Mills Compounds, Shakti Mills Lane, Mahalaxmi, Mumbai – 400011. Tel: (022) 24968011/12. FAX: (022) 24973085.
E-mail: info@bluelabelindia.com ; Website: www.bluelabelindia.com

5. Es Em Inc
1, Kaiser Bagh Avenue, Lucknow – 226 001. Tel: (0522) 2217173 / 2225997. FAX: (0522) 2271760. E-mail:Manocha_2k@sify.com.

6. HiMedia Laboratories Ltd.
A-406 Bhaveshwar Plaza, LBS Marg, Mumbai – 400086. Tel: (022) 5003747 / 5000970 / 5001607 / 5000653. FAX: (022) 5005764/5002468/5002286.
Website: www.himedialabs.com; E-mail: info@himedialabs.com

10. Ranbaxy Fine Chemicals Ltd.
25, Nehru Place, New Delhi – 110109. Website: www.ranbaxyfinechemicls.com

11. Qualigens Fine Chemicals Division
Glaxo India Ltd., Dr. Anne Besant Road, Worli, Mumbai – 400025. Tel: (022) 4939753/4933871. E-mail: inqfccustomercare@gsk.com

12. Spectrochem Pvt. Ltd.
1129 Navivan CHS Ltd., Mumbai – 400008. FAX: (022) 2059299.

13. Kinetic Imaging Ltd.
Indian Distributor: Es Em Inc., 1, Kaiser Bagh Avenue, Lucknow – 226 001. Tel: (0522) 2217173 / 2225997. FAX: (0522) 2271760. E-mail: Manocha_2k@sify.com.

14. Techno Source
l-21, Stone Castle, Mandapeshwar, Borivli (West), Mumbai– 400103, Maharastra, Tel: 022-28937286, Fax: 022-28959781, Email: ts1@vsnl.com

15. GIBCO BRL, Life Technologies
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