

Tech Tip: Single Cell Gel Electrophoresis (Comet) Assay

Overview

The Single Cell Gel Electrophoresis assay (SCGE), or Comet Assay, measures DNA damage from individual cells based on the migration of denatured DNA through an electrophoretic field^(1,2) (Fig. 1). Individual cells or nuclei are embedded in agarose, lysed to expose their DNA, alkali treated to denature/relax the DNA, and electrophoresed to separate the DNA.^(3,4) The DNA is visualized by EtBr, silver stain, or fluorescent dyes. Damaged DNA containing strand breaks migrates farther in the gel than intact DNA, creating an image resembling a celestial comet. When performed under alkaline conditions, SCGE detects (but does not distinguish between) double-stranded breaks, single-stranded breaks, alkali-labile sites (single-stranded breaks), incomplete excision repair (single-stranded breaks), DNA-DNA interactions, and DNA-protein interactions. The specificity of SCGE can be enhanced to investigate specific types of DNA damage by adding DNA modifying enzymes. Enzymes which have successfully been used in the comet assay include Mut M (Fpg) for oxidative damage, AlkA for alkylation damage, Endo III for oxidized pyrimidines, and Proteinase K for DNA-protein interactions (see Table I: Enzymes to Study DNA).

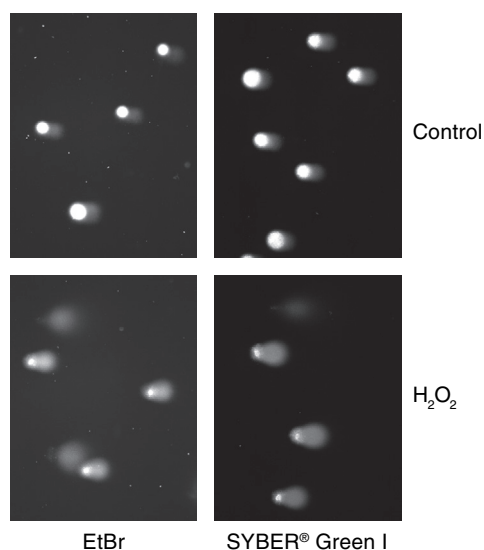


Fig. 1. Single Cell Gel Electrophoresis performed on Mouse NIH/3T3 cells incubated with Mut M (Fpg) under Control and severe DNA damage (5% hydrogen peroxide) conditions.

The genotoxic effects of virtually any chemical or industrial compound can be investigated using SCGE. Analyses can include any organism, tissue, or culture from which a single cell or nuclei suspension can be isolated. Resulting DNA comets are analyzed visually by estimating the extent of DNA damage or measuring tail length. Image analysis tools are available to analyze various parameters of the comets including tail moment, percent migrated DNA, and tail length (for more information, see Ref. 9). USB offers reliable nuclease-free reagents to maximize reproducibility and accuracy of the SCGE assay as well as various DNA repair, recombination, and replication enzymes to extend the utility of the SCGE assay (see Table II).

Table I. Enzymes to Study DNA:

Enzyme	DNA recognition site	Action
Mut M FPG (PN 71430)	8-oxoguanine, 8-oxoadenine, formamidopyrimidines (FapyA, FapyG, methyl-fapy-guanine, aflatoxin B ₁ -fapy-guanine), 5-hydroxy-cytosine, 5-hydroxy-uracil, ring-opened N-7guanine adducts (7-methylguanine), and AP sites	Excises altered base and nicks DNA backbone ^(5,6)
Endonuclease IV (Endo IV) (PN 78340)	AP sites	Nicks DNA backbone
Mut Y (PN 71428)	G/A mispairs	Excises A, creates AP site
Exonuclease III (Exo III) (PN 70023Y)	AP sites	Nicks DNA backbone
Proteinase K (PN 76230Y)	Protein DNA interactions	Degrades protein ⁽⁵⁾
Uracil DNA Glycosylase, <i>E. coli</i> (PN 71960Y) Uracil DNA Glycosylase, Heat-Labile (PN 78310)	Uracil in DNA	Excises U, creates AP site
T4 Endonuclease VII (PN 78300)	Mismatches, insertion/deletion loops, gaps, AP sites	Nicks DNA backbone
AlkA (3-methyl-adenine DNA glycosylase)	N ³ - and N ⁷ -Alkyl purines, O ² -alkyl pyrimidines, hypoxanthine, xanthosine, 5-formyluracil, oxanine	Excises altered base, creates AP site ^(5,7)
hOGG1	8-oxoguanine paired with C, 8-oxoadenine paired with C, FapyG, methyl-FapyG, AP site paired with C	Excises altered base and nicks DNA backbone
UVDE (Ultraviolet DNA Glycosylase)	6-4 dipyrimidine photoproducts, pyrimidine dimers, AP sites	Nicks 5' of altered bases or AP site
T4 Endonuclease V (T4-PDG)	Cyclobutane pyrimidine dimers	Excises 5' pyrimidine and nicks DNA backbone
Endonuclease III	Thymine glycol, dihydrothymine, dihydroxydihydrothymine, uracil glycol, urea	Excises altered base and nicks DNA backbone ⁽⁵⁾
TDG	G/T or G/U mispairs 3,N ⁴ -ethenocytosine	Excises T or U, creates AP site
UvrABC Endonuclease	Pyrimidine dimers, psoralen monoadducts and cross-links, 2-acetylaminofluorene monoadducts	Removes section of DNA with the altered base ⁽⁸⁾

Table II. Ultrapure Reagents from USB for SCGE:

	Product No.
Agarose - Separation ≥ 500 bp, Genetic Performance Certified™	75817
PBS, 10X Solution, pH 7.4	75889
Agarose - Low Melt, Separation ≤1000 bp, Genetic Performance Certified™	32829
Triton® X-100	22686
EDTA, 0.5M Solution	15694
TBE Buffer, 5X Solution	75891
TE Buffer, 1X Solution	75893
TE Buffer, 50X Solution	75834
Tris-HCl, 1M Solution, pH 7.5	22639
Ethidium Bromide Drops	75816
Sodium Chloride, 5M Solution	75888
Tris	75825
Water, Nuclease-Free	71786

Buffer Preparation:

Lysis Solution:

	For 1 L
2.5M NaCl	500 ml 5M NaCl (PN 75888)
0.1M EDTA	200 ml 0.5M EDTA, pH 8.0 (PN 15694)
10mM Tris-HCl, pH 10	1.2 gm/L Tris (PN 75825)
	Adjust pH to 10.0 with NaOH pellets.
	H ₂ O (PN 71786) to 1 L

Filter through a 0.5 µm filter. Chill to 4°C for convenience in assay.

Before use add Triton X-100 (PN 22686) to 1% and DMSO to 10% (optional). Addition of Triton X-100 will turn solution slightly cloudy. DMSO prevents oxidation during lysis incubation.

Denaturation Solution (make fresh daily):

	For 1 L
300mM NaOH	12 gm NaOH
1mM EDTA	2 ml 0.5M EDTA (PN 15694)
	H ₂ O (PN 71786) to 1 L

pH should be >13

Exposure of cells to genotoxic substances:

Genotoxic substances should be treated with extreme care. Cells can be exposed while in culture by adding directly to the growth media or while embedded in agarose on the slide during the SCGE assay. Chemicals, preferably dissolved in PBS, can be applied to the slides by adding 50 µl of solution per section (half) of the microscope slide and dispersing with a cover slip, or alternatively slides can be immersed in the chemical solution (*e.g.* hydrogen peroxide). Incubate on ice to inhibit the endogenous DNA repair enzymes.

Single Cell Gel Electrophoresis Assay Protocol

Pre-coating of Glass Microscope Slides:

1. Dip a glass microscope slide into 1.5% molten agarose (PN 75817) made up in distilled/filtered water. Immediately wipe all agarose off the back of the slide.
2. Place slide on bench top or in drying oven and allow the agarose to dry completely. Slides can be used for up to 1 week when stored at RT.

Embedding of Cells:

3. Centrifuge cells (*e.g.* cells grown in culture or trypsinized cells from plates) at 2000 x g in a microcentrifuge for 1 min at 4°C, discard the supernatant.
4. Resuspend cells in 1 ml of 1X PBS (10X PBS, PN 75889).
5. Centrifuge cells at 100 x g in a microcentrifuge for 5 min at 4°C, discard the supernatant.
6. Resuspend cells in PBS to about 25000 cells per ml.
7. Mix 5 µl of cell suspension (about 125 cells) with 35 µl 0.8% Agarose, Low Melt (PN 32829) (0.8% LM agarose can be microwaved to liquefy and stored at 37°C until use). Pipette mixture immediately onto half a microscope slide and cover with a 22 x 22 mm cover slip.
8. Repeat application of cells for second half of slide.
9. Place slides on ice or at 4°C for 5 min to allow agarose to form gel.
10. Remove cover slips.
11. Cells are now accessible for additional manipulation (*e.g.* exposure to UV irradiation or to chemical agents).

Lysis of Cells:

12. Cover slides in staining trays with ice cold Lysis Solution. (Note: Triton X-100 lyses the cells. High salt removes histones from DNA.)
13. Incubate on slow shaker for 1 hr at 4°C. Lysis times may vary depending on the cells being used.
14. Rinse slides with filtered water.
15. Wash slides in filtered water 3 times for 5 min each wash.

Treatment of Slides with DNA Modifying Enzymes (Optional, Mut M example):

16. Dilute Mut M (Fpg) 1×10^2 to 10^3 in reaction buffer (PN 71430).
17. Add 50 μ l of enzyme solution per half of microscope slide. Cover with a cover slip.
18. Incubate at 37°C for 30 min.
19. Remove cover slip.

Denaturation/DNA Unwinding:

20. Cover slides with Denaturation Solution.
21. Incubate with slow shaking for 20 min at 4°C.

Electrophoresis:

22. Pre-equilibrate slides by covering with 1X TBE (5X TBE, PN 75891) buffer for 10 min. Place in horizontal submarine gel electrophoresis unit.
23. Electrophorese samples in 1X TBE. For a 13 cm electrode distance, electrophorese for 3-1/2 min at 25V, 3mA. Vary time of electrophoresis as appropriate.
24. Wash slides in filtered water 2 times for 5 min each wash.
25. Slides can be stained immediately or dried completely for later use. For drying, slides can be dipped in 100% EtOH (or MeOH) and dried at RT or 37°C.

Electrophoresis Alternative: Slides can be electrophoresed in fresh Denaturing Solution at 25 volts for 10 min for ss and ds DNA breaks. Electrophoresis time may vary. Neutralize in 0.4M Tris, pH 7.5 (1M Tris, pH 7.5, PN 22639)

Visualization of DNA:

SYBR® Green I Nucleic Acid Stain - Dilute SYBR Green I stock 1:10,000 in TE Buffer (pH 7.5-8.0) as directed by manufacturer. Pipet 100 μ l diluted SYBR Green I onto dry slides and add cover slips. Visualize under fluorescence microscope.

Ethidium Bromide - Pipette 80 μ l (2 drops) of 2 μ g/ml EtBr (PN 75816) onto dry slide and apply cover slip. Incubate for 30 min at room temperature. Remove cover slips and dip slides in filtered H₂O to rinse. Visualize under fluorescence microscope.

Silver Stain - Silver stained slides have the advantage of visualization on a light microscope and slides can be stored for future reference. For detailed protocol, see reference 10.

Troubleshooting:**No comets on Control or Experimental slides.**

1. Cells not lysed.
 - Extend incubation time in lysis buffer.
2. Lysis buffer not effective.
 - Test alternative lysis buffers.
3. DNA not electrophoresed.
 - Increase voltage.
 - Increase electrophoresis time.
4. DNA not fully denatured.
 - Increase denaturing time.

All cells, Control and Experimental, give comets.

1. DNA damage.
 - Use chilled buffers, substitute Hank's Balanced Salt Solution (HBSS) for PBS
 - Shorten time between cell harvest and start of assay.
 - Reduce cell exposure to fluorescent light in work area.
 - 0.8% LM agarose was too hot, cool to 37°C.
2. Electrophoretic field too high.
 - Reduce electrophoretic time.
 - Reduce voltage.

Agarose lifting off of slide.

1. Too much agitation during washes/incubation steps.
 - Reduce agitation rate of shaker.
 - Perform washes and incubations on the bench top without agitation.
2. Hydrated for too long.
 - Dry slides completely before staining DNA (after electrophoresis).

Comets on a slide vary widely in tail length and orientation of tail.

1. Current through the gel was inconsistent because the gel layer was not uniform.
 - Use cover slips when dispensing the agarose + cells onto the slide to create a uniform gel layer.

Comets near edges of gel layer do not match comets on rest of slide.

An 'edge effect' in the assay. Do not score comets along edge of gel layer.

Plant Nuclei Comet Assay Procedure⁽¹¹⁾:

Additional Supplies:

10% SDS - PN 77504

1. Treat leaves with UV, H₂O₂ or other.
2. Wash 4 small leaves in distilled water.
3. Place leaves in a petri dish with 3 ml cold PBS + 1 mM EDTA, slice the leaves with a razor blade into small pieces.
4. Combine 15 µl of leaf extract with 100 µl 0.8% Agarose, Low Melt, mix, pipette onto half a pre-coated microscope slide (See "Precoating of Glass Microscope Slides" in above protocol). Cover with cover slip immediately.
Note: Amount of leaf extract suspended in agarose can be varied to alter density of comets on the slide.
5. Repeat for second half of slide.
6. Place on ice for 5 min to solidify.
7. Remove cover slips.
8. Cover slides in Plant Lysis Solution (2.5% SDS in TBE Buffer) chilled to 4°C.
9. Incubate on slow shaker for 1 hr at 4°C.
10. Rinse slides with filtered water.
11. Wash the slides in filtered water for 10 min. Repeat rinse and wash steps.
12. Follow "Treatment of Slides with DNA Modifying Enzyme", "Denaturation/DNA Unwinding", "Electrophoresis", "Electrophoresis Alternative", and "Visualization of DNA" as stated for cell lines.

References

1. OSTLING, O., AND JOHANSON, K. J. (1984) *Biochemical and Biophysical Research Communications* **12**, 291-298.
2. SINGH, N. P., MCCOY, M. T., TICE, R. R., AND SCHNEIDER E. L. (1988) *Experimental Cell Research* **175**, 184-191.
3. TICE, R. R., AGURELL, E., ANDERSON, D., BURLINSON, B., HARTMANN, A., KOBAYASHI, H., MIYAMAE, Y., ROJAS, E., RYU, J. C., AND SASAKI Y. F. (2000) *Environmental and Molecular Mutagenesis* **35**, 206-221.
4. HARTMANN, A., AGURELL, E., BEEVERS, C., BRENDLER-SCHWAAB, S., BURLINSON, B., CLAY, P., COLLINS, A., SMITH, A., SPEIT, G., THYBAUD, V., AND TICE, R. R. (2003) *Mutagenesis* **18**, 45-51.
5. WOZNIAK, K. AND BLASIAK J. (2003) *Mutation Research* **535**, 127-139.
6. SPEIT, G., SCHÜTZ, P., BONZHEIM, I., TRENZ, K., AND HOFFMANN, H. (2004) *Toxicology Letters* **146**, 151-158.
7. COLLINS, A. R., DUSINSKÁ, M., AND HORSKÁ, A. (2001) *Acta Biochimica Polonica* **48**, 611-614.
8. COLLINS, A. R., DOBSON, V. L., DUSINSKA, M., KENNEDY, G., AND STETINA, R. (1997) *Mutation Research* **375**, 183-193.
9. Comet assay references, procedures and information can be found at two websites:
A) Comet Assay Interest Group (NIH) <http://cometassay.com/>
B) Comet Assay Forum (Industrial Toxicology Research Center, Lucknow, India) <http://www.comet.itrcindia.org/> - Dhawan, A., Bajpayee, M., Pandey A. K., and Parmar, D., The SCGE/Comet Assay Protocol, ITRC.
10. NADIN, S. B., VARGAS-ROIG, L. M., AND CIOCCA D. R. (2001) *The Journal of Histochemistry & Cytochemistry* **49**, 1183-1186.
11. KOPPEN, G. (1997) *Comet Newsletter*, Kinetic Imaging Ltd., **6**, 2-3.

©2006 USB Corporation—All rights reserved.

USB and the logo are registered trademarks of USB Corporation.

Triton is a registered trademark of Union Carbide Chemicals and Plastics Company.

Uracil DNA Glycosylase - A license may be required from Invitrogen Corporation to practice the process claimed in U.S. Patent No. 5,035,996.

SYBR is a registered trademark of Molecular Probes, Inc.

USB Corporation
26111 Miles Road, Cleveland, Ohio 44128
www.usbweb.com

