CometAssay®
Reagent Kit for
Single Cell Gel Electrophoresis Assay
Catalog # 4250-050-K

I. Background

Trevigen's CometAssay®, or single cell gel electrophoresis assay, provides a simple and effective method for evaluating DNA damage in cells. The principle of the assay is based upon the ability of denatured, cleaved DNA fragments to migrate out of the nucleoid under the influence of an electric field, whereas undamaged DNA migrates slower and remains within the confines of the nucleoid when a current is applied. Evaluation of the DNA "comet" tail shape and migration pattern allows for assessment of DNA damage. The Neutral CometAssay® is typically used to detect double-stranded breaks, whereas the Alkaline CometAssay® is more sensitive, and is used to detect smaller amounts of damage including single and double-stranded breaks.

Trevigen's CometAssay® uses our exclusive CometSlide™ that is specially treated to promote adherence of low melting point agarose. This eliminates the time consuming and unreliable traditional method of preparing base layers of agarose. The use of Trevigen's CometSlide™ shortens assay time and allows the rapid and reliable analysis of large numbers of samples. In comet assays, cells are immobilized in a bed of low melting point agarose, on a Trevigen CometSlide™. Following gentle cell lysis, and for the Alkaline CometAssay®, samples are treated with alkali to unwind and denature the DNA and hydrolyze sites of damage. For both assays, cells are lysed and the remaining nucleoids are subjected to electrophoresis and subsequent staining with a fluorescent DNA intercalating dye. Trevigen recommends using CometAssay® Control Cells (cat# 4256-010-CC) when performing alkaline electrophoresis, and Neutral CometAssay® Control Cells (cat# 4257-010-NC) when performing the neutral comet assay, to monitor assay conditions and verify reproducibility between separate runs. Trevigen also provides SYBR® Green I1 for DNA visualization by epifluorescence microscopy, providing improved sensitivity compared to ethidium bromide. As an alternative for researchers who do not have access to a fluorescence microscope, silver staining allows standard light microscopy for comet tail analysis. We recommend the use of Trevigen's CometAssay® Electrophoresis System (cat# 4250-050-ES) designed to eliminate known causes of assay variability. The electrophoresis step is performed using an Alkaline Electrophoresis Solution pH > 13, for the alkaline version, whereas a Neutral Electrophoresis Buffer is recommended for the neutral version. Quantitative and statistical data can readily be generated by analysis of the results using one of several commercially available image analysis software packages which calculate tail length, percent DNA in the tail, and tail moment.

Gentaur Molecular Products
Voortstraat 49
1910 Kampenhout, Belgium

The CometAssay® may be coupled with Trevigen's FLARE™ (Fragment Length Analysis using Repair Enzymes) Assay that provides the added ability to probe for specific types of DNA damage using DNA repair glycosylases. Contact Trevigen for more details about analysis of DNA damage and repair. 1 SYBR® Green I is a registered product of Molecular Probes, Eugene OR, and is sold under license from Molecular Probes, Inc. Please see p.18 for complete licensing terms. Use of this reagent outside of the scope of these terms is not endorsed by Trevigen,Inc.

II. Precautions and Limitations

1. For Research Use Only. Not for use in diagnostic procedures.
2. The physical, chemical, and toxicological properties of the products contained within the CometAssay® Kit may not have been fully investigated. Therefore, Trevigen recommends the use of gloves, lab coats, and eye protection while using any of these chemical reagents. Trevigen assumes no liability for damage resulting from handling or contact with these products.
3. Lysis Solution contains 1% sodium lauryl sarcosinate which is an irritant and precipitates with long term storage at 4°C. In case of eye or skin contact, wash thoroughly under running water. In case of ingestion, rinse mouth with water and seek medical advice.
4. SYBR® Green I contains DMSO. Please refer to MSDS sheets.

III. Materials Supplied

Component Catalog # Amount Storage
Lysis Solution 4250-050-01 2 x 500 ml Room temp.
Comet LMAgarose (LMA) 4250-050-02 15 ml 4°C
Trevigen CometSlide™ 4250-050-03 25 each Room temp.
200 mM EDTA, pH 10 4250-050-04 12.5 ml Room temp.
SYBR® Green I nucleic acid gel stain 4250-050-05 5 µl -20°C

IV. Materials/Equipment Required But Not Supplied

Equipment:
1. 1–20 µl, 20–200 µl, 200–1,000 µl pipettors, and tips
2. Serological pipettor and pipets
3. Boiling water bath and 37°C water bath
4. Horizontal electrophoresis apparatus (CometAssay® ES cat# 4250-050-ES)*
5. Epifluorescence microscope equipped with Fluorescein filter or light transmission microscope when using silver staining kit.
6. 1 L graduated cylinder
7. 4°C refrigerator/cold room

Reagents:

1. Deionized water
2. 10X PBS, Ca++ and Mg++ free***** (cat# 4870-500-6)
3. 95% Ethanol (reagent grade)
4. TE Buffer (10 mM Tris (pH 7.5), 1 mM EDTA)

For alkaline assays:
5. NaOH Pellets
6. 0.5 M EDTA (pH 8.0)

For neutral assays:
7. 10X Neutral Electrophoresis Buffer
8. Ammonium Acetate
9. Sodium Acetate
10. Glacial Acetic Acid

Optional reagents:
11. Silver staining kit***** (cat# 4254-050-K)
12. Dimethylsulfoxide
13. Tris Borate EDTA Buffet

*****Available from Trevigen.

V. Reagent Preparation

Reagents marked with an asterisk (*) should be prepared immediately before use. Wear gloves, lab coat and eye protection when handling any chemical reagents.

1. 1X PBS, Ca++ and Mg++ free
Dilute 10X PBS with deionized water to prepare 1X PBS. Store at room temperature. (10X PBS is available from Trevigen, cat# 4870-500-6.)

2. Lysis Solution
For up to 10 slides (2 samples per slide) prepare: Lysis Solution (cat # 4250-050-01) 40 ml DMSO (optional) 4 ml Chill at 4°C, or on ice, for at least 20 minutes before use. The addition of DMSO is optional and is required only for samples containing heme, such as blood cells or tissue samples. The buffer formulation is proprietary.

3. Comet LMAgarose
The Comet LMAgarose is ready to use once molten. Loosen the cap to allow for expansion then heat the bottle in a 90–100°C water bath for 5 minutes, or until the agarose is molten (Caution: Microwaving is not recommended). Place the bottle in a 37°C water bath for at least 20 minutes to cool. The LMAgarose will remain molten at 37°C for sample preparation indefinitely. The LMAgarose formulation is proprietary.

4. SYBR® Green I Staining Solution
Prepare SYBR® Green I Staining Solution from the SYBR® Green I concentrate provided (10,000X concentrate in DMSO). SYBR® Green I (cat # 4250-050-05) 1 µl TE Buffer, pH 7.5 10 ml (TE: 10 mM Tris-HCl pH 7.5, 1 mM EDTA) The diluted stock is stable for several weeks when stored at 4°C in the dark.

5. Anti-fade Solution (optional)
Prepare if fading of samples occurs. In a 50 ml tube, mix until dissolved: p-Phenylenediamine dihydrochloride 500 mg 1X PBS 4.5 ml Add approximately 400 µl of 10 N NaOH drop wise with stirring until pH of solution reaches 7.5-8.0. Add 1X PBS to increase the volume to 5 ml, and 45 ml of glycerol for a final volume of 50 ml. Vortex mixture thoroughly and apply 10 µl per sample, covering samples with coverslip. Nail polish may be used to seal coverslip. Restaining of slides is not recommended. Anti-fade solution is stored at -20°C for one month. Darkening of solution may occur.

For Alkaline Comet Assay:

6. Alkaline Unwinding Solution, pH>13 (200 mM NaOH, 1 mM EDTA)

Wear gloves when preparing and handling the Alkaline Unwinding Solution. Per 50 ml of Alkaline Solution combine:
NaOH Pellets 0.4 g
200 mM EDTA (cat # 4250-050-04) 250 µl
dH2O 49.75 ml
Stir until fully dissolved. The solution will warm during preparation. Allow to cool to room temperature before use.

7. Alkaline Electrophoresis Solution pH >13 (200 mM NaOH, 1 mM EDTA) for the CometAssay® ES system:

Prepare a stock solution of 500 mM EDTA, pH 8.
For 1 liter of electrophoresis solution:
NaOH pellets 8 g
500 mM EDTA, pH 8 2 ml
dH2O (after NaOH is dissolved) q.s. to: 1 liter
Use of freshly made solution is recommended. Prechill at 4°C.

For Neutral Comet Assay:

8. 1X Neutral Electrophoresis Buffer

To prepare 10X **Neutral Electrophoresis Buffer**:
Tris Base (mol. wt. = 121.14) 60.57 g
Sodium Acetate (mol. wt. = 136.08) 204.12 g
Dissolve in 450 ml of dH2O. Adjust to pH = 9.0 with glacial acetic acid. Adjust volume to 500 ml and filter sterilize. Store at room temperature. Dilute the 10X stock to 1X in dH2O to prepare 1 liter working strength buffer and prechill at 4°C.

9. DNA Precipitation Solution

Prepare a 10 ml stock solution of 7.5M Ammonium Acetate:
NH4Ac (mol. wt. = 77.08) 5.78 g
dH2O (after NH4Ac is dissolved) add to: 10 ml
For 50 ml of DNA precipitation solution combine:
7.5 M NH4Ac (mol. wt. = 77.08) 6.7 ml
95% EtOH (reagent grade) 43.3 ml

VI. Sample Preparation and Storage

Cell samples should be prepared immediately before starting the assay, although success has been obtained using cryopreserved cells (see below). Cell samples should be handled under dimmed or yellow light to prevent DNA damage from ultraviolet light. Buffers should be chilled to 4°C or on ice to inhibit endogenous damage occurring during sample preparation and to inhibit repair in the unfixed cells. PBS must be calcium and magnesium free to inhibit endonuclease activities. The appropriate controls should also be included (see below). Optimal results in the CometAssay® are usually obtained with 500–1000 cells per CometSlide**TM** sample area. Using 50 µl of a cell suspension at 1 x 105 cells per ml combined with 500 µl of LMAgarose will provide the correct agarose concentration and cell density for optimal results when spreading 50 µl per well.

Suspension Cells

Cell suspensions are harvested by centrifugation. Resuspend cells at 1 x 105 cells/ml in ice cold 1X PBS (Ca++ and Mg++ free). The media used for cell culture can reduce the adhesion of the agarose on the CometSlide™.

Adherent Cells

Gently scrape cells using a rubber policeman. Transfer cells and medium to centrifuge tube, perform cell count, and then pellet cells. Wash once in ice cold 1X PBS (Ca++ and Mg++ free). Resuspend cells at 1 x 105 cells/ml in ice cold 1X PBS (Ca++ and Mg++ free).

Tissue Preparation

Place a small piece of tissue into 1–2 ml of ice cold 1X PBS (Ca++ and Mg++ free),b 20 mM EDTA. Using small dissecting scissors, mince the tissue into very small pieces and let stand for 5 minutes. Recover the cell suspension, avoiding transfer of debris. Count cells, pellet by centrifugation, and resuspend at 1 x 105 cells/ml in ice cold 1X PBS (Ca++ and Mg++ free). For blood rich organs (*e.g.*, liver, spleen), chop tissue into large pieces (1–2 mm3), let settle for 5 minutes then aspirate and discard medium. Add 1–2 ml of ice cold 20 mM EDTA in 1X PBS (Ca++ and Mg++ free), mince the tissue into very small pieces and let stand for 5 minutes. Recover the cell suspension, avoiding transfer of debris. Count cells, pellet, and resuspend at 1 x 105 cells/ml in ice cold 1X PBS (Ca++ and Mg++ free).

Controls

A sample of untreated cells should always be processed to control for assay variability, endogenous levels of damage within cells, and for additional damage that may occur during sample preparation. Control cells and treated cells should be handled in an identical manner. If UV damage is being studied; the cells should be kept in low level yellow light during processing. Trevigen offers two sets of suspension cell preparations containing different levels of DNA damage to standardize methods between individual users, different runs, and laboratories for alkaline (cat# 4256-010-CC) and neutral (cat# 4257-010-NC) electrophoresis conditions, respectively.

Note: To generate samples positive for comet tails, treat cells with 100 µM hydrogen peroxide or 25 µM KMnO4 for 20 minutes at 4°C. Treatment will generate significant oxidative damage in the majority of cells, thereby providing a positive control for each step in the alkaline comet assay.

Method for Cryopreservation of Cells Prior to CometAssay®

Certain cells, *e.g.* lymphocytes, may be successfully cryopreserved prior to performing CometAssay® (Visvardis *et al.*). A pilot study should be performed to determine if cryopreservation is appropriate for the cells in use.

1. Centrifuge cells at 200 x g for 5 minutes.
2. Resuspend cell pellet at 1 x 107 cells/ml in 10% (v/v) dimethylsulfoxide,b 40% (v/v) medium, 50% (v/v) fetal calf serum.
3. Transfer aliquots of 2 x 106 cells into freezing vials.
4. Freeze at -70°C with -1°C per minute freezing rate.
5. Recover cells by submerging in 37˚C water bath until the last trace of ice has melted.
6. Transfer to 15 ml of prechilled 40% (v/v) medium, 10% (w/v) dextrose, 50% (v/v) fetal calf serum.
7. Centrifuge at 200 x g for 10 minutes at 4°C.
8. Resuspend in ice cold 1X PBS (Ca++ and Mg++ free) and proceed with CometAssay®.

VII. Assay Protocol

The electrophoresis conditions used will determine the sensitivity of the assay. Neutral CometAssay® will detect double-stranded DNA breaks, whereas Alkaline CometAssay® will detect single and double-stranded DNA breaks, and the majority of abasic sites as well as alkali labile DNA adducts (*e.g.* phosphoglycols, phosphotriesters). The comet assay has been reported to detect DNA damage associated with low doses (0.6 cGy) of gamma irradiation, providing a simple technique for quantitation of low levels of DNA damage. Prior to performing the comet assay, a viability assay should be performed to determine the dose of the test substance that gives at least 75% viability. False positives may occur when high doses of cytotoxic agents are used. For cryopreservation of cells, fixing the CometSlide™ samples, and storage, refer to Section VI:

Sample Preparation and Storage.
The Alkaline CometAssay® requires approximately 2–3 hours to complete, whereas the Neutral CometAssay® requires 4 hours, including the incubations and electrophoresis. Once the cells or tissues have been prepared the procedure is not labor intensive. The Lysis Solution may be chilled and the LMAgarose melted while the cell and tissue samples are being prepared.

A. Alkaline CometAssay®

1. Prepare Lysis Solution (see Section V: *Reagent Preparation*) and chill at 4°C or on ice for at least 20 minutes before use.
2. Melt LMAgarose in a beaker of boiling water for 5 minutes, with the cap loosened. Place bottle in a 37°C water bath for at least 20 minutes to cool. The temperature of the agarose is critical or the cells may undergo heat shock. Heat blocks are not recommended for regulating the temperature of the agarose.
3. Combine cells at 1 x 105/ml with molten LMAgarose (at 37°C) at a ratio of 1:10 (v/v) and immediately pipette 50 µl onto CometSlide™. If necessary, use side of pipette tip to spread agarose/cells over sample area to ensure complete coverage of the sample area. When working with many samples it may be convenient to place aliquots of the molten agarose into prewarmed microcentrifuge tubes and place the tubes at 37°C. Add cells to one tube, mix by gently pipetting once or twice, then transfer 50 µl aliquots onto each sample area as required. Then proceed with the next sample of cells
Comet LMAgarose (molten and at 37°C from step 2) 500 µl
Cells in 1X PBS (Ca++ and Mg++ free) at 1 x 105/ml 50 µl
Note: If sample is not spreading evenly on the slide, warm the slide at 37°C
before application.
4. Place slides flat at 4°C in the dark (*e.g.* place in refrigerator) for 10 minutes. A 0.5 mm clear ring appears at edge of CometSlide™ area. Increasing gelling time to 30 minutes improves adherence of samples in high humidity
environments.
5. Immerse slides in prechilled Lysis Solution and leave on ice, or at 4°C, for 30 minutes to 60 minutes.
6. Drain excess buffer from slides and immerse in freshly prepared Alkaline Unwinding Solution, pH>13 (see Section V: *Reagent Preparation*). WEAR
GLOVES WHEN PREPARING OR HANDLING THIS SOLUTION.
7. Leave CometSlide™ in Alkaline Unwinding Solution for 20 to 60 minutes at room temperature, in the dark.
8. For the CometAssay® ES tank, add 950 ml prechilled Alkaline Electrophoresis Solution, place slides in electrophoresis slide tray and cover with Slide Tray Overlay. Set power supply to 21 volts and apply voltage for 30 minutes. (If not using an ES unit, see Appendix B.)
9. Gently drain excess electrophoresis solution, immerse twice in dH2O for 5 minutes each, then in 70% ethanol for 5 minutes.
10.Dry samples at ≤ 45°C for 10-15 minutes. Drying brings all the cells in a single plane to facilitate observation. Samples may be stored at room temperature, with desiccant prior to scoring at this stage.
Note: Trevigen offers the CometAssay® Silver Staining Kit designed for comet staining (Cat # 4254-200-K). Silver staining allows visualization of comets on any transmission light microscope and permanently stains the samples for archiving and long term storage. It is recommended that samples be dried before silver staining.
11.Place 100 µl of diluted SYBR® Green I (See Section V: *Reagent*

Preparation) onto each circle of dried agarose and place in refrigerator for 5 minutes. Gently tap slide to remove excess SYBR solution. Allow slides to dry completely at room temperature in the dark.
12.View slides by epifluorescence microscopy. (SYBR® Green I's maximum excitation and emission are respectively 494 nm/521 nm. Fluorescein filter is adequate).

B. Neutral CometAssay®

1. Prepare Lysis Solution (see Section V: *Reagent Preparation*) and chill at 4°C or on ice for at least 20 minutes before use.
2. Melt LMAgarose in a beaker of boiling water for 5 minutes, with the cap loosened, and then cool in a 37°C water bath for at least 20 minutes.
3. Combine cells at 1 x 105/ml with molten LMAgarose (at 37°C) at a ratio of 1:10 (v/v) and immediately pipette 50 µl onto CometSlide™. Use side of pipette tip to spread agarose/cells over sample area.
Comet LMAgarose (molten and at 37°C from step 2) 500 µl
Cells in 1X PBS (Ca++ and Mg++ free) at 1 x 105/ml 50 µl
Note: If sample is not spreading evenly on the slide, warm the slide at 37 °C before application.
4. Place slides flat at 4°C in the dark (*e.g.* place in refrigerator) for 10 minutes. A 0.5 mm clear ring appears at edge of CometSlide™ area. Increasing gelling time to 30 minutes improves adherence of samples in high humidity environments.
5. Immerse slides in prechilled (Step 1) Lysis Solution and leave on ice or at 4°C for 1 hour.
6. Remove slides from Lysis Buffer, drain excess buffer from slide and wash slide by immersing in 50 ml of prechilled 1X Neutral Electrophoresis Buffer for 30 minutes at 4°C (see Section V: *Reagent Preparation*).
7. For the CometAssay® ES tank, add 950 ml prechilled 1X Neutral Electrophoresis Buffer, place slides in electrophoresis slide tray and cover with Slide Tray Overlay. Set power supply to 21 volts and apply voltage for 1 hour at 4°C. For other electrophoresis units, align slides equidistant from electrodes, add 1X Neutral Electrophoresis Buffer not to exceed 0.5 cm above slides, and apply voltage at 1 volt per cm (measured electrode to electrode).
8. Drain excess Neutral Electrophoresis Buffer and immerse slides in DNA Precipitation Solution for 30 minutes at room temperature.
9. Immerse slides in 70% ethanol for 30 minutes at room temperature.
10.Dry samples at ≤ 45°C for 10-15 minutes. Drying brings all the cells in a single plane to facilitate observation. Samples may be stored at room temperature, with desiccant prior to scoring at this stage.

Note: Trevigen offers the CometAssay® Silver Staining Kit designed for comet staining (Cat # 4254-200-K). Silver staining allows visualization of comets on any transmission light microscope and permanently stains the samples for archiving and long term storage. It is recommended that samples be dried before silver staining.
11.Place 100 µl of diluted SYBR® Green I onto each sample for 30 minutes. Gently tap slide to remove excess SYBR solution. Allow slide to dry completely at room temperature in the dark.
12.View slide by epifluorescence microscopy using (Fluorescein filter is adequate). (SYBR® Green I's maximum excitation and emission are respectively 494 nm/521 nm.)

VIII. Data Analysis

When excited (425–500 nm) the DNA-bound SYBR® Green I emits green light. In healthy cells the fluorescence is confined to the nucleoid (comprised of high molecular weight DNA):

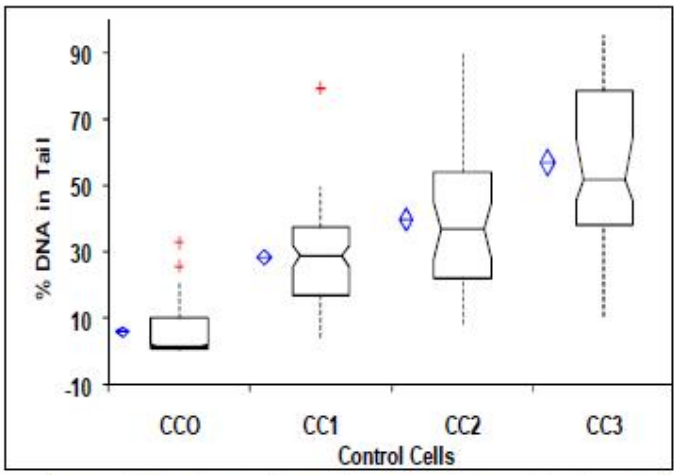
undamaged DNA is supercoiled and thus, does not migrate very far out of the nucleoid under the influence of an electric current. Whereas in cells that have accrued DNA damage, migrating fragments (comet tail) from the nucleoid (comet head) are observed. The negatively charged DNA migrates toward the anode and the extrusion length reflects increasing relaxation of supercoiling, which is indicative of damage. Common descriptors of DNA damage for alkaline comet assays are Percent DNA in the Tail, and Tail Moment. Percent DNA in the Tail is a normalized measure of the percent of total cell DNA found in the tail. Tail moment is a damage measure combining the amount of DNA in the tail with distance of migration. In neutral comet assays, Tail Moment is primarily used, since tail length continues to increase in contrast to alkaline comet tails which have finite lengths. Qualitative Analysis (Alkaline **CometAssay®**) The comet tail can be scored according to DNA content (intensity). The control (untreated cells) should be used to determine the characteristics of data for a healthy cell. Scoring can then be made according to nominal, medium or high intensity tail DNA content. At least 50 cells should be scored per sample. Quantitative Analysis (Alkaline and Neutral **CometAssay®**) There are several image analysis systems that are suitable for quantitation of CometAssay® data. The more sophisticated systems include the microscope, camera and computer analysis package. These systems can be set up to measure the length of DNA migration, image length, nuclear size, and calculate DNA damage parameters. At least 50 randomly selected cells should be analyzed per sample. A list of commercially available software package is available from Trevigen.

Featured Data:

Alkaline CometAssay®

In Figure 1a, data collected for each alkaline CometAssay® Control Cell population (cat# 4256-010-CC) is shown as side-by side vertical box plots for comparison. The diamond shows the mean and confidence interval around the mean. The notched box shows the median, lower and upper quartiles, and the 75% confidence interval around the median. An example is provided below.

Figure 1a: Box-Whisker plot of Control Cells: Percent DNA in Comet Tail



% DNA by Etoposide	n	Mean	SD	SE	75% CI of Mean	Median	IQR	75% CI of Median
CCO	50	5.757	7.7270	1.0928	4.485 to 7.029	1.640	8.925	1.290 to 2.230
CC1	50	28.374	14.0080	1.9810	26.068 to 30.680	28.990	20.313	25.180 to 31.840
CC2	50	39.736	21.8164	3.0853	36.144 to 43.328	37.050	32.183	27.790 to 44.630
CC3	50	56.800	23.5893	3.3360	52.916 to 60.683	51.905	40.240	45.460 to 64.390

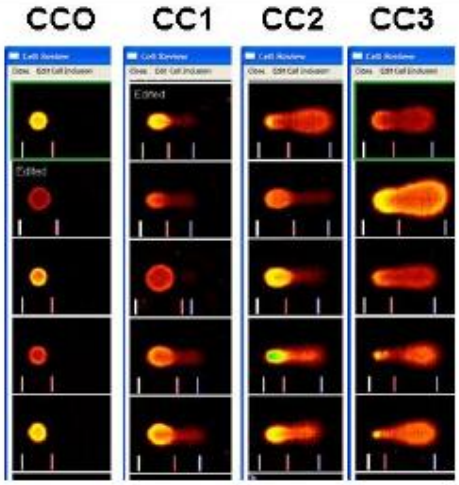
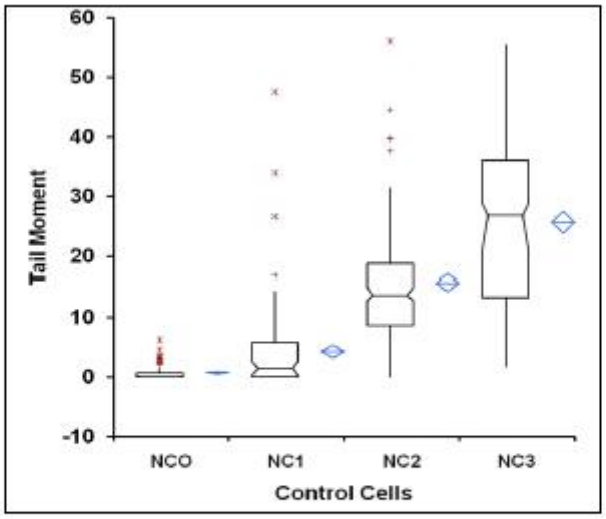


Figure 1b: Examples of comet tails for each population.

Neutral CometAssay®

Data collected for each Neutral CometAssay® Control Cell population (cat# 4257-010-NC) is provided below.

Figure 2a: Box-Whisker plot of Neutral Control Cells: Tail Moment



TM by Bleomycin	n	Mean	SD	SE	75% CI	Median	IQR	75% CI
NCO	75	0.677	1.2410	0.1433	0.511 to 0.843	0.000	0.637	0.000 to 0.140
NC1	75	4.316	7.7817	0.8986	3.274 to 5.358	1.360	5.748	0.240 to 2.510
NC2	75	15.711	10.7829	1.2451	14.268 to 17.155	13.600	10.117	12.830 to 14.950
NC3	75	25.730	13.7918	1.5925	23.884 to 27.577	26.780	22.750	20.810 to 28.930

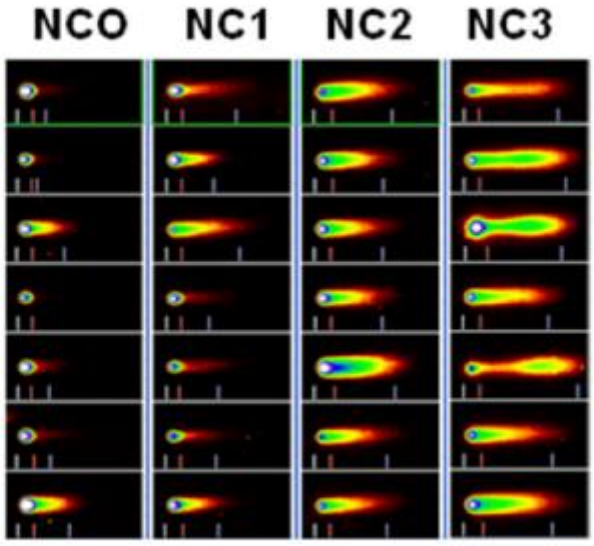


Figure 2b: Example comet tails for each population.

IX. References

1. Lemay, M. and K.A. Wood, 1999. Detection of DNA damage and identification of UVinduced photoproducts using the CometAssay® kit. *BioTechniques* **27**(4):846-51.

2. Angelis, K.J., M. Dusinska and A.R. Collins. 1999. Single cell gel electrophoresis: Detection of DNA damage at different levels of sensitivity. *Electrophoresis* **20:**2133-38.

3. Morris, E.J., J.C. Dreixler, K-Y. Cheng, P.M. Wilson, R.M. Gin and H.M. Geller. 1999. Optimization of single-cell gel electrophoresis (SCGE) for quantitative analysis of neuronal DNA damage. *BioTechniques* **26:**282-9.

4. Malyapa, R.S., C. Bi, E.W. Ahern, J.L. Roti, 1998. Detection of DNA damage by the alkali comet assay after exposure to low dose gamma radiation. *Radiation Res* 1**49:**396-400.

Gentaur Molecular Products
Voortstraat 49
1910 Kampenhout, Belgium

5. Henderson, L., A. Wolfreys, J. Fedyk, C. Bourner, S. Windeback, 1998. The ability for the comet assay to discriminate between genotoxins and cytotoxins. *Mutagenesis* **13:**89-94.
6. Visvardis, E.E., A.M. Tassiou, S.M. Piperakis, 1997. Study of DNA damage induction and repair capacity of fresh cryopreserved lymphocytes exposed to H2O2 and γ-irradiation with the alkaline comet assay. *Mutation Res* **383:**71-80.
7. Fairbairn, D.W., P.L. Olive, K.L. O'Neill, 1995. The comet assay: a comprehensive review. *Mutation Res* **339:**37-59.
8. Collins, A.R., A.G. Ma, S.J. Duthie, 1995. The kinetics of repair of oxidative DNA damage (strand breaks and oxidized pyrimidine dimers) in human cells. *Mutation Res* **336:**69-77.
9. Singh, N.P., M.T. McCoy, R.R. Tice, E.L. Schneider, 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res* **175:**184-91.
10. Singh, N.P., R.E. Stephens, 1997. Microgel electrophoresis: sensitivity, mechanisms, and DNA electrostretching. *Mutation Res* **383:**167–175.
11. Östling, O., K. J. Johanson, 1984. Microelectrophoretic study of radiation-induced DNA damage in individual cells. *Biochem Biophys Res Commun* **123:**291-8.

X. Related Products Available From Trevigen

Contact Trevigen for details of our unique product line for studying DNA damage and repair. All of Trevigen's kits include highly qualified enzymes, substrates, buffers, full instructions for use, and a synopsis specific for your kit.

CometAssay® Kits:

4250-050-ESK CometAssay® Starter Kit each
4250-050-ES CometAssay® ES each
4251-050-K CometAssay® Silver Kit 50 samples
4254-200-K CometAssay® Silver Staining Kit 200 samples
4252-040-K CometAssay® Higher Throughput Kit 40 samples
4253-096-K CometAssay® Kit 96 Wells 96 samples
PARP Assay Kits:

4684-096-K HT Colorimetric PARP/Apoptosis Assay 96 tests
4685-096-K HT Chemiluminescent PARP /Apoptosis Assay 96 tests
4520-096-K HT PARP in vivo Pharmacodynamic Assay II 96 tests

FLARE™ Assay Kits:

Catalog #	Description	Damage Recognized	Size
4040-100-FK 4040-100-FM	Fpg Kit	8-oxoguanine, DNA containing formamidopyrimidine moieties	75 samples 100 samples
4055-100-FK 4055-100-FM	T4-PDG Kit	Cis-syn isomers of cyclo-butane pyrimidine dimers	75 samples 100 samples
4065-100-FK 4065-100-FM	cv-PDG Kit	Cis-syn and trans-syn isomers of cyclobutane pyrimidine dimers	75 samples 100 samples
4130-100-FK 4130-100-FM	hOGG1 Kit	8-oxoguanine, DNA containing formamidopyrimidine moieties	75 samples 100 samples
4100-100-FK 4100-100-FM	UVDE Kit	Cyclobutane pyrimidine dimers, (6-4) photoproducts	75 samples 100 samples
4045-01K-FK 4045-01K-FM	Endonuclease III Kit	Thymine Glycol, 5,6-dihydro-thymine, urea, 5-hydroxy-6-hydrothy-mine, 5,6-dihydro-uracil, alloxan, 5-hydroxy-6-hydrouracil, uracil glycol, 5-hydroxy-5-methylhy-dantoin, 5-hydroxycytosine,5-hydroxy-uracil, methyl-tartonylurea, thymine ring saturated or fragmentation product	75 samples 100 samples

DNA Damage Antibodies:

Catalog #	Description	Size
4411-PC-100	γ-H2AX	100 µl
4410-PC-100	Fen-1	100 µl
4350-MC-100	UVssDNA	100 µg
4354-MC-50	anti-8-oxo-dG monoclonal	50 µl

Accessories:

Catalog #	Description	Size
4256-010-CC	CometAssay® Control Cells (alkaline assay)	1 set
4257-010-NC	Neutral CometAssay® Control Cells	1 set
4250-050-03	CometSlide™ (2 well)	25 slides
4252-200-01	CometAssay® HT Slide (20 well)	10 slides
4253-960-03	96 Well CometSlide™	10 slides
3950-300-02	FLARE™ Slides	100 slides
4040-100-FM	Fpg FLARE™ Module	>100 samples
4130-100-FM	hOGG1 FLARE™ Module	>100 samples
4045-100-FM	Endonuclease III FLARE™ Module	>100 samples
4055-100-FM	T4-PDG FLARE™ Module	>100 samples
4065-100-FM	cv-PDG FLARE™ Module	>100 samples
3950-075-SP	FLARE™ Sample Prep	>100 samples
4100-050-FM	UVDE FLARE™ Module	>100 samples
4370-096-K	HT 8-oxo-dG ELISA Kit	96 wells

XI. Appendices

Appendix A
Neutral CometAssay®

The CometAssay® may be performed using neutral conditions that employ 1X TBE. Without treatment with Alkaline Buffer, this Neutral CometAssay® will also detect mainly double-stranded breaks.

1. Prepare Lysis Solution (see Section V: *Reagent Preparation*) and chill at 4°C or on ice for at least 20 minutes before use.
2. Melt LMAgarose in a beaker of boiling water for 5 minutes, with the cap loosened, and then cool in a 37°C water bath for at least 20 minutes.
3. Combine cells at 1 x 105/ml with molten LMAgarose (at 37°C) at a ratio of 1:10 (v/v) and immediately pipette 50 µl onto CometSlide™. Use side of pipette tip to spread agarose/cells over sample area. Comet LMAgarose (molten and at 37°C from step 2) 500 µl Cells in 1X PBS (Ca++ and Mg++ free) at 1 x 105/ml 50 µl

Note: If sample is not spreading evenly on the slide, warm the slide at 37 °C before application.
4. Place slides flat at 4°C in the dark (*e.g.* place in refrigerator) for 10 minutes. A 0.5 mm clear ring appears at edge of CometSlide™ area. Increasing gelling time to 30 minutes improves adherence of samples in high humidity environments.
5. Immerse slides in prechilled (Step 1) Lysis Solution and leave on ice or at 4°C for 30 minutes.
6. Remove slides from Lysis Buffer, drain excess buffer from slide and wash slide by immersing in 50 ml of 4°C 1X TBE buffer for 15 minutes. To prepare 10X TBE: Tris Base 108 g Boric Acid 55 g EDTA (disodium salt) 9.3 g Dissolve in 900 ml dH2O. Adjust volume to 1 liter and filter sterilize. Store at room temperature. Dilute the 10X TBE to 1X in dH2O to prepare 1 liter working strength buffer and prechill at 4°C.
7. For the CometAssay® ES tank, add 4°C 950 ml 1X TBE Buffer, place slides in electrophoresis slide tray and cover with Slide Tray Overlay. Set Power supply to 21 volts and apply voltage for 40 minutes.

Note: For other electrophoresis units, align slides equidistant from electrodes, add 1X TBE Buffer not to exceed 0.5 cm above slides, and apply voltage at 1 volt per cm (measured electrode to electrode).
8. Drain excess TBE, immerse slides in dH2O for 5 minutes.
9. Immerse slides in 70% ethanol for 5 minutes.
10.Dry samples at ≤ 45 oC for 10-15 minutes. Drying brings all the cells in a single plane to facilitate observation. Samples may be stored at room temperature, with desiccant prior to scoring at this stage.
11.Place 100 µl of diluted SYBR® Green I onto each sample and place in refrigerator for 5 minutes. Gently tap slide to remove excess SYBR solution. Allow slide to dry completely at room temperature in the dark.
12.View slide by epifluorescence microscopy using (Fluorescein filter is adequate). (SYBR® Green I's maximum excitation and emission are respectively 494 nm/521 nm.)

Appendix B

Instructions for alkaline comet assay with other electrophoresis units.
Since the Alkaline Electrophoresis Solution is a non-buffered system, **temperature control is highly recommended.** In-house testing has shown great temperature fluctuations when conducting the alkaline electrophoresis at ambient temperature. To improve temperature control, the use of a large electrophoresis apparatus (20–30 cm between electrodes) is recommended. Performing the electrophoresis at cooler temperatures (*e.g.* 4°C) will diminish background damage, increase sample adherence at high pHs and significantly improves reproducibility. Choose the method that is most convenient for your laboratory and always

use the same conditions, CometAssay® Control Cells (cat# 4256-010-CC), power supplies and electrophoresis chambers for comparative analysis.

Alternative Reagents:

1. Alkaline Unwinding Solution, pH>13 (300 mM NaOH, 1 mM EDTA)

Wear gloves when preparing and handling the Alkaline Unwinding Solution. Per 50 ml of Alkaline Solution combine: NaOH Pellets 0.6 g 200 mM EDTA (cat # 4250-050-04) 250 µl dH2O 49.75 ml Stir until fully dissolved. The solution will warm during preparation. Allow to cool to room temperature before use.

2. Alkaline Electrophoresis Solution pH >13 (300 mM NaOH, 1 mM EDTA) for other electrophoresis systems:

Prepare a stock solution of 500 mM EDTA, pH 8.
For 1 liter of electrophoresis solution:
NaOH pellets 12 g
500 mM EDTA, pH 8 2 ml
dH2O (after NaOH is dissolved) q.s. to: 1 liter
Adjust the volume prepared based on the dimensions of your electrophoresis apparatus. Use of freshly made solution is recommended. Prechill at 4°C.
Align slides equidistant from electrodes and carefully add 300 mM NaOH (1 mM EDTA) Alkaline Solution until level just covers samples. Set the voltage to about 1 Volt/cm. Add or remove buffer until the current is approximately 300 mA and perform electrophoresis for 20–40 minutes. Continue at step 9 on page 7.

Appendix C

Detection of (6-4)-dipyrimidine UV Adducts Using Anti-UVssDNA Antibody

The anti-UVssDNA antibody (cat # 4350-MC-100) can be use to detect (6-4)- dipyrimidine photoproducts, directly in the comets. The Alkaline CometAssay® is performed on the samples as indicated in Section VII: *Assay Protocol*. The staining step is omitted; samples are fixed with ethanol, dried and treated as follows:

1. Cover dried sample area with 10 µg/ml of anti-UVss DNA antibody diluted in 1X PBST, 1% BSA, (PBST: 1X PBS, 0.05% Tween® 20) and incubate overnight at 4°C.
2. Wash sample 3 times with 1X PBST for 5 minutes each.
3. Incubate with secondary antibody conjugate, *e.g.* anti-mouse IgG (H+L) biotin-conjugated or fluorescein-conjugated.
4. Wash sample 3 times with PBST for 5 minutes each.
5. If a biotinylated secondary antibody was used, incubate with streptavidinfluorescein (cat # 4800-30-14) diluted 1:300 in 1X PBS (or streptavidin coupled to fluorophore of choice).
6. Wash sample 3 times with PBST for 5 minutes each.
7. View under fluorescence microscope equipped with appropriate filters.
8. Counterstaining is possible using a dye that does not interfere with the fluorophore.

XII. Troubleshooting Guide

General Problems

PROBLEM	CAUSE	ACTION
Unexpected and/or variety of tail shape.	LMAgarose too hot	Cool LMAgarose to 37°C before adding cells.
Cells in LMAgarose did not remain attached to the CometSlide™.	Electrophoresis solution too hot.	Control temperature performing electrophoresis at 4°C.
	Cells were not washed to remove medium before combining with LMAgarose.	The pH of medium and carry over serum proteins, etc., can reduce the adherence of the agarose. Resuspended cells in 1X PBS.
	Agarose percentage was too low.	Do not increase ratio of cells to molten agarose by more than 1 to 10.
	LMAgarose was not fully set before samples were processed.	Ensure 0.5 mm dried ring due to agarose disc retraction is seen at the edge of the CometSlide™ area.
	LMAgarose unevenly set on the slide.	Spread the agarose with the side of a pipette tip to ensure uniformity of agarose disc and better adherence.
	Rinsing steps too harsh.	Gently place slides into solutions. Do not pour solutions over slides.

Specific to Alkaline Comet Assay

PROBLEM	CAUSE	ACTION
Majority of cells in untreated control sample have large comet tails.	Unwanted damage to cells occurred in culture or in sample preparations	Check morphology of cells to ensure healthy appearance.
		Handle cells or tissues gently to avoid physical damage.
	Electrophoresis solution too hot	Control temperature by performing electrophoresis at 4°C.
	Intracellular activity	Keep cells on ice and prepare cell samples immediately before combining with molten LMAgarose.
Majority of cells in untreated control sample have small to medium comet tails.	Endogenous oxidative damage or endonuclease activity after sample preparation is damaging DNA.	Ensure Lysis solution was chilled before use.
		Add DMSO to any cell sample that may contain heme groups.
		Ensure PBS used is calcium and magnesium free.
		Work under dimmed light conditions or under yellow light.
In positive control (*e.g.* 100 µM hydrogen peroxide for 30 minutes on ice) no evidence of comet tail.	No damage to DNA.	Use fresh hydrogen peroxide to induce damage.
	Sample was not processed correctly.	Ensure each step in protocol was performed correctly. Failure to lyse, denature in alkali, or to properly perform electrophoresis may generate poor results.

PROBLEM	CAUSE	ACTION
Comet tails present but not significant in positive control.	Insufficient denaturation in Alkaline Solution. Insufficient electrophoresis time.	Increase time in Alkaline Solution up to 1 hour. Increase time of electrophoresis up to up to 1 hour for alkaline electrophoresis. Increase time of electrophoresis when running at cold temperatures.

Specific to Neutral Comet Assay

PROBLEM	CAUSE	ACTION
In positive control no evidence of comet tail.	Damaging agent doesn't cause double-strand breaks.	Confirm damage by Alkaline Comet. Run Neutral Control Cells to confirm electrophoresis conditions. Increase treatment with damaging agent.
In positive control comet tails are extremely long and do not fit analysis window.	Cells are necrotic or apoptotic. Electrophoresis time too long.	Verify 75% viability. Decrease treatment with damaging agent. Decrease electrophoresis time to 15-30 minutes.

SYBR® Green I nucleic acid gel stain licensing terms:

This product is sold under license from Molecular Probes, Inc. under US Patents Nos. 5,436,134 and 5,658,751 for use in a comet assay for internal research and development only, where research and development use expressly excludes the use of this product for providing medical, diagnostic or any other testing analysis or screening services or providing clinical information or clinical analysis, in return for compensation on a per-test basis, and research and development use expressly excludes incorporation of this product into another product for commercialization even if such other product would be commercialized for research and/or development use.