TREVIGEN® Instructions

For Research Use Only. Not For Use In Diagnostic Procedures

CometAssay™ Silver

Reagent Kit for Single Cell Gel Electrophoresis Assay and Silver Staining

Catalog # 4251-050-K

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Table of Contents

I. Background

Trevigen provides a simple and effective method for evaluating DNA damage in cells by combining the comet assay (also called single cell gel electrophoresis) and silver staining. The assay principle is based upon the ability of denatured, cleaved DNA fragments to migrate out of the cell under the influence of an electric field, whereas undamaged DNA migrates more slowly and remains within the confines of the nucleoid. Evaluation of the DNA "comet" tail shape and migration pattern allows for assessment of DNA damage. In this assay, cells are immobilized in a bed of low melting point agarose, on a Trevigen CometSlide™. Following gentle cell lysis, samples are treated with alkali to unwind and denature the DNA and hydrolyze sites of damage. The samples are then submitted to electrophoresis. Trevigen's **CometAssay™ Silver** kit provides all the reagents for silver staining of the processed CometSlide™ allowing visualization by standard light microscopy and providing permanent staining for sample archiving.

The electrophoresis step may be performed using TBE buffer or alkaline electrophoresis solution. TBE circumvents the problems associated with use of non-buffered alkali for electrophoresis, such as poor DNA migration due to saturation of charge by sodium ions and difficulties in controlling voltage. TBE is preferred for analysis of apoptosis and enables use of the tail length, rather than the tail moment, for data analysis. The alkaline electrophoresis is more sensitive and will detect smaller amounts of damage. The electrophoresis time may be extended up to 40 minutes when running at very low amperage. Data may be analyzed qualitatively if the comets are scored according to categories of small to large tail lengths. 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 and tail moment.

Trevigen's **CometAssay™ Silver** kit 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 CometSlides shortens assay time and allows the rapid and reliable analysis of large numbers of samples. Contact Trevigen if fluorescent DNA visualization is preferred, several options providing improved sensitivity compared to ethidium bromide are available. 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.

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™ Silver 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. 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. The Silver Staining reagents contains small quantities of hazardous materials: 2.8% Formaldehyde is found in Cat# 4254-200-02 and 10% tungstosilicic acid is found in Cat# 4254-200-03. Please consult the MSDS sheets for details.

III. Materials Supplied

CometAssay Reagents	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.
Silver Staining Reagents			
10X Fixation Additive	4254-200-05	2.2 ml	Room temp.
20X Staining Reagent #1	4254-200-01	1.2 ml	Room temp.
20X Staining Reagent #2	4254-200-02	1.2 ml	Room temp.
20X Staining Reagent #3	4254-200-03	1.2 ml	Room temp.
*2X Staining Reagent #4	4254-200-04	1.2 g	Room temp.

*Resuspend in 12 ml dH_2O and store at 4 °C. Stable for 3 months after resuspension.

IV. Materials/Equipment Required But Not Supplied

Equipment:

1. 1-20, 20-200, 200-1000 µl pipettors, and tips
2. Serological pipettor and pipets
3. Boiling water bath and 37 °C water bath
4. Horizontal electrophoresis apparatus
5. Light transmission microscope
6. 1 L graduated cylinder
7. 4 °C refrigerator
8. Peristaltic pump for recirculation of buffer (optional).

Reagents:

1. 10X PBS, Ca^{2+} and Mg^{2+} free* (cat# 4870-500-6)
2. NaOH Pellets
3. Dimethylsulfoxide (DMSO)(required only for heme rich samples)
4. 10X TBE Buffer (optional)
5. Ethanol
6. 0.5 M EDTA (pH 8.0)(required for alkaline electrophoresis)
7. Deionized water
8. Methanol
9. Glacial acetic acid
10. High quality deionized water (dH_2O). For best results, use 18 mΩ dH_2O for all steps requiring water during the staining process.

*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^{2+} and Mg^{2+} free**
   Dilute 10X PBS with deionized water to prepare 1X PBS. Store at room temperature.

2. **Lysis Solution**
   For up to 10 slides (2 samples per slide) prepare:

Lysis Solution (Cat# 4250-050-01)	40 ml
DMSO	4 ml (optional)

   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.

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.

4. **Alkaline Solution, pH>13**
   Wear gloves when preparing and handling the Alkaline Solution.
   Per 50 ml of Alkaline Solution combine:

NaOH Pellets	0.6 g
200 mM EDTA (Cat# 4250-050-04)	250 µl
dH_2O	49.75 ml

   Stir until fully dissolved. The solution will warm during preparation. Allow to cool to room temperature before use.

5. **Electrophoresis Solution**
   Prepare one of the following electrophoresis solutions based on the sensitivity of assay desired.

   A. 1X TBE Electrophoresis Buffer
   To prepare 10X TBE:

Tris Base	108 g
Boric Acid	55 g
EDTA (disodium salt)	9.3 g

   Dissolve in 900 ml of dH_2O. Adjust volume to 1 liter and autoclave. Store at room temperature. Dilute the 10X TBE to 1X in deionized water to prepare working strength buffer.

   Or B. Alkaline Electrophoresis Solution pH >13 (300 mM NaOH, 1 mM EDTA) Wear gloves when preparing and handling the Alkali Solution
   Prepare stock solutions of 500 mM Na_2EDTA, pH 8.0:

NaOH pellets	12 g
500 mM EDTA, pH 8.0	2 ml
dH_2O (after NaOH is dissolved)	to 1 liter

   Adjust the volume prepared based on the dimensions of your electrophoresis apparatus. Use of freshly made solution is recommended.

6. **Silver Staining Solutions**
   A. Fixation solution
   Prepare immediately before fixation. Mix per sample:

10X Fixation Additive (Cat# 4254-200-05)	10 µl
dH_2O	30 µl
methanol	50 µl
glacial acetic acid	10 µl

   B. 2X Staining Reagent #4 (Cat# 4254-200-04)
   Before first use, add 12 ml of dH_20 to bottle, stir until dissolved. Store at 4 °C, prewarm to room temperature before each use.

   C. Staining solution (prepare immediately before staining)
   The staining reagents 1, 2 and 3 are ready to use in the staining solution as described here:
   Per sample, mix in a microtube:

dH_2O	35 µl
20X Staining Reagent #1 (Cat# 4254-200-01)	5 µl
20X Staining Reagent #2 (Cat# 4254-200-02)	5 µl
20X Staining Reagent #3 (Cat# 4254-200-03)	5 µl

   Mix well by tapping tube and add 50 µl 2X Staining Reagent #4* (Cat# 4254-200-04) (*prepared in section V.6.B.) For 10 samples:

dH_2O	350 µl
20X Staining Reagent #1 (Cat# 4254-200-01)	50 µl
20X Staining Reagent #2 (Cat# 4254-200-02)	50 µl
20X Staining Reagent #3 (Cat# 4254-200-03)	50 µl

   Mix by tapping tube and add 500 µl 2X Staining Reagent #4* (Cat# 4254-200-04) (*prepared in section V.6.B.)

D. Stop solution
Prepare a 5% acetic acid solution. 100 µl per sample area is required.

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™ sample area. Using 50 µl of a cell suspension at 1 x 10^5 cells per ml combined with 500 µl of LMAgarose will provide the correct agarose concentration and cell density for optimal results when plating 75 µl per sample.

A. Suspension Cells
Cell suspensions are harvested by centrifugation. Resuspend cells at 1 x 10^5 cells/ml in ice cold 1X PBS (Ca^{2+} and Mg^{2+} free). The media used for cell culture can reduce the adhesion of the agarose on the CometSlide™.

B. Adherent Cells
Gentle trypsinization of adherent cells is compatible with the comet assay. Alternatively, gently scrape cells using a rubber policeman. Transfer cells and medium to centrifuge tube, perform cell count, then pellet cells. Wash once in ice cold 1X PBS (Ca^{2+} and Mg^{2+} free). Resuspend at 1 x 10^5 cells/ml in ice cold 1X PBS (Ca^{2+} and Mg^{2+} free).

C. Tissue Preparation
Place a small piece of tissue into 1-2 ml of ice cold 1X PBS (Ca^{2+} and Mg^{2+} free), 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 10^5 cells/ml in ice cold 1X PBS (Ca^{2+} and Mg^{2+} free). For blood rich organs (e.g., liver, spleen), chop tissue into large pieces (1-2 mm^3), let settle for 5 minutes then aspirate and discard medium. Add 1-2 ml of ice cold 20 mM EDTA in 1X PBS (Ca^{2+} and Mg^{2+} 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.

D. Controls
A sample of untreated cells should always be processed to control for endogenous levels of damage within cells, and for 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. If you require a sample that will be positive for comet tails, treat cells with 100 µM hydrogen peroxide or 25 µM $KMnO_4$ for 10 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 comet assay. Note that the dimensions and characteristics of the comet tail, as a consequence of H_2O_2 or $KMnO_4$ treatment, may be different to those induced by the damage under investigation.

E. 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 10^7 cells/ml in 10% (v/v) dimethylsulfoxide, 40% (v/v) medium, 50% (v/v) fetal calf serum.
3. Transfer aliquots of 2 x 10^6 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^{2+} and Mg^{2+} free) and proceed with comet assay.

VII. Assay Protocol

A. Comet Assay
Both protocols provided are for alkaline unwinding conditions. Electrophoresis conditions will determine the sensitivity of the assay. TBE electrophoresis or Neutral electrophoresis after alkaline unwinding will detect single-stranded DNA breaks, double-stranded DNA breaks, and may detect apurinic and apyrimidinic sites. Alkaline electrophoresis will detect single-stranded and double stranded DNA breaks, and the majority of apurinic and apyrimidinic sites, as well as alkali labile DNA adducts (e.g. phosphoglycols, phosphotriesters). The comet assay reportedly detects 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 information on performing the neutral comet assay that will predominantly detect double-stranded DNA breaks, see Section XIII: *Appendix A*. For cryopreservation of cells, fixing the CometSlide™ samples, and storage, refer to Section VI: *Sample Preparation and Storage*.

Trevigen's CometAssay™ requires approximately 2-3 hours to complete, 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.

All steps are performed at room temperature unless otherwise specified. Work under dimmed or yellow light to prevent damage from UV.

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 10^5/ml with molten LMAgarose (at 37 °C) at a ratio of 1: 10 (v/v) and immediately pipette 75 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 micro-centrifuge tubes and place the tubes at 37 °C. Add cells to one tube, mix by gently pipetting once or twice, then transfer 75 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^{2+} and Mg^{2+} free) at 1 x 10^5/ml	50 µl

Note: If sample is not spreading evenly on the slide, warm the slide at 37 °C before application.

4. Place slide flat at 4 °C in the dark (e.g. place in refrigerator) for 10 min. 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 slide in prechilled Lysis Solution and leave on ice or at 4 °C for 30 to 60 minutes.

6. Tap off excess buffer from slide 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 Alkali Unwinding Solution for 20 to 60 minutes at room temperature in the dark.

To perform TBE Electrophoresis go to step 8, or for Alkaline go to step 13.

For TBE Electrophoresis

8. Remove slide from Alkaline Solution, gently tap excess buffer from slide and wash by immersing in 1X TBE buffer for 5 minutes, 2 times (see Section V: *Reagent Preparation*).

9. Transfer slide from 1X TBE buffer to a horizontal electrophoresis apparatus. Place slides flat onto a gel tray and align equidistant from the electrodes. Pour 1X TBE buffer until level just covers samples. Set power supply to 1 volt per cm (measured electrode to electrode). Apply voltage for 10 minutes.

10. Gently tap off excess TBE, and dip slide in 70% ethanol for 5 minutes.

11. Air dry samples. Drying brings all the cells in a single plane to facilitate observation. Samples may be stored at this stage. Store at room temperature with desiccant.

12. Proceed to section B. *Silver Staining*

For Alkaline Electrophoresis

13. Transfer slide from Alkaline Solution to a horizontal electrophoresis apparatus. Place slides flat onto a gel tray and align equidistant from the electrodes. Carefully pour the 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.

Tips:
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 alkaline electrophoresis at ambient temperature. To improve temperature control, the use of a large electrophoresis apparatus (25-30 cm between electrodes) is recommended, along with recirculation of the electrophoresis solution. Alternatively, performing the electrophoresis at cooler temperatures (e.g. 16 °C or 4 °C) will diminish background damage, increase sample adherence at high pHs and significantly improve reproducibility. Choose the method that is most convenient for your laboratory and always use the same conditions, power supplies and electrophoresis chambers for comparative analysis.

14. Gently tap off excess electrophoresis solution, rinse by dipping several times in dH_2O, then immerse slide in 70% ethanol for 5 minutes.

15. Air dry samples. 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.

16. Proceed to section B. *Silver Staining*.

B. Silver Staining

1. Cover the sample area with 100 µl of Fixation solution prepared in section V.6.A, *Reagent Preparation*.

2. Incubate for 20 minutes at room temperature.

3. Rinse in dH_2O for 30 minutes.

4. Cover sample area with 100 µl of Staining Solution prepared in section V.6.C. *Reagent Preparation*.

5. Incubate at room temperature for 5 to 20 minutes. (Intensity of staining can be visualized under the microscope using 10X objective, and reaction stopped when comets are easily visible).

6. Stop reaction by covering samples with 100 µl of 5% acetic acid and incubate for 15 minutes.

7. Rinse in dH_2O.

8. Air dry and store in the dark.

VIII. Warning/Safety

The final Silver Staining solution (prepared in section V, step 6 C.) is considered hazardous material. Disposal should be performed according to local and state regulations. It is recommended to tap solution off the slide into a container for safe disposal.

IX. Data Analysis and Troubleshooting

Silver Staining of DNA generates a brown to black stain easily detectable by microscopy. In healthy cells, the DNA is confined to the nucleoid: undamaged DNA is supercoiled and thus does not migrate very far under the influence of an electric current. In cells that have accrued damage to the DNA, the alkali treatment unwinds the DNA, releasing fragments that migrate from the nucleoid when subjected to an electric field. The negatively charged DNA migrates toward the anode and the extrusion length reflects increasing relaxation of supercoiling, which is indicative of damage.

When using TBE as the electrophoresis buffer, the length of the comet tail may be correlated with DNA damage. When using alkaline electrophoresis conditions, the distribution of DNA between the tail and the head of the comet should be used to evaluate the degree of DNA damage. The characteristics of the comet tail including length, width, and DNA content may also be useful in assessing qualitative differences in the type of DNA damage.

A. Qualitative Analysis
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 75 cells should be scored per sample.

B. Quantitative Analysis
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 establish the length of DNA migration, image length, nuclear size, and calculate the tail moment (Lee *et al.*). At least 75 randomly selected cells should be analyzed per sample. A list of commercial software packages is available from Trevigen.

X. Troubleshooting Guide

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 recirculating the electrophoresis solution or performing the assay at less than 20 °C.
	Intracellular activity.	Keep cells on ice and prepare cell samples immediately before combining with molten LMAgarose.
	LMAgarose too hot.	Cool LMAgarose to 42 °C before adding cells.
Majority of cells in the 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 (optional), or to properly perform electrophoresis may generate poor results.
Comet tails present but not significant in positive control.	Insufficient denaturation in Alkaline Solution.	Increase time in Alkaline Solution up to 1 hour.
	Insufficient electrophoresis time	Increase time of electrophoresis up to 20 minutes for TBE and up to 1 hour for alkaline electrophoresis. Increase time of electrophoresis when running at cold temperature.
Cells in LMAgarose did not remain attached to the CometSlide™.	Electrophoresis solution too hot.	Control temperature by recirculating the electrophoresis solution or performing the assay at less than 20 °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. Resuspend 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.

XI. References

1. Lemay, M. and K.A. Wood, 1999. Detection of DNA damage and identification of UV-induced photoproducts using the CometAssay™ kit. BioTechniques **27**: 846-851.

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**:2133-2138.

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**:282-289.

4. Malyapa, R.S., C. Bi, E.W. Ahern, and J.L. Roti Roti, 1998. Detection of DNA damage by the alkali comet assay after exposure to low dose gamma radiation. Radiation Res **149**:396-400.

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, and S.M. Piperakis,1997. Study of DNA damage induction and repair capacity of fresh cryopreserved lymphocytes exposed to H_2O_2 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, and 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, and E.L. Schneider, 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res **175**:184 -191.

11. Black J.A. 1985. A silver stain for isoelectric focusing in agarose gel and its application for analysing unconcentrated cerebrospinal fluid. Electrophoresis **6**:27-29.

12. Delincee H. 1997. Silver staining of DNA in the "comet assay". Comet Newsletter (6). Kinetic Imaging Inc. Liverpool, UK

13. Lee E., E. Oh, J. Lee, D. Sul, and J. Lee, 2004. Use of the tail moment of the lymphocytes to evaluate DNA damage in human biomonitoring studies. Toxicol Sci 81:121-132.

XII. 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:

Catalog #	Description	Size
4252-040-K	CometAssay™ HT	40 samples
4250-050-K	CometAssay™ Kit	50 samples
4253-096-K	CometAssay™ Kit 96 Wells	96 samples

PARP Assay Kits:

Catalog #	Description	Size
4667-50-K	PARP Activity Assay Kit	50 tests
4677-096-K	HT Universal Colorimetric PARP Assay w/ Histone Coated Strip Wells	96 samples
4676-096-K	Universal Chemiluminescent PARP Assay w/Histone Coated Strip Wells	96 samples
4667-250-01	Recombinant Human PARP Enzyme	250 µl
4668-100-1	Recombinant Human PARP (High Specific Activity)	1000 Units

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
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
4130-100-FK 4130-100-FM	hOGG1 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
4100-100-FK 4100-100-FM	UVDE Kit	Cyclobutane pyrimidine dimers, (6-4) photoproducts	75 samples 100 samples

DNA Damage Antibodies:

Catalog #	Description	Size
4410-PC-100	Fen-1	100 µl
4411-PC-100	γ-H2AX	100 µl
2372-PC-050	p53 Ack317	50 µl
2370-PC-050	p53 Ack379	50 µl
2371-PC-050	p53 Ack382	50 µl
2381-PC-100	p53 total	100 µl
4350-MC-100	UVssDNA	100 µg
4431-MC-100	XPF	100 µg
4421-MC-100	XRCC1	100 µg
4354-MC-50	anti-8-oxo-dG monoclonal	50 µl

Accessories:

Catalog #	Description	Size
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
4100-050-FM	UVDE FLARE™ Module	>100 samples
3950-075-SP	FLARE™ Sample Prep	>100 samples

XIII. Appendices

Appendix A

Neutral CometAssay™

The CometAssay™ may be performed using neutral conditions. Without treatment with Alkaline Buffer, the Neutral CometAssay™ will detect mainly double-stranded breaks and can be useful for assessing the DNA fragmentation associated with apoptosis.

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, then cool in a 37 °C water bath for at least 20 minutes.

3. Combine cells at 1 x 10^5/ml with molten LMAgarose (at 37 °C) at a ratio of 1:10 (v/v) and immediately pipette 75 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^{2+} and Mg^{2+} free) at 1 x 10^5/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 slide in prechilled (Step 1) Lysis Solution and leave on ice or at 4 °C for 30 minutes.

6. Remove slide from Lysis Buffer, tap excess buffer from slide and wash slide by immersing in 50 ml of 1X TBE buffer (see Section V: *Reagent Preparation*).

7. Transfer slides from 1X TBE buffer, and place flat onto a gel tray submerged in 1X TBE buffer in a horizontal electrophoresis apparatus. Align slides equidistant from the electrodes. Set power supply to 1 volt per cm (measured electrode to electrode). Apply voltage for 10-20 minutes.

8. Tap off excess TBE, rinse slides briefly in dH_2O.

9. Immerse slide in 70% ethanol for 5 minutes

10. Air dry samples. 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. Proceed to section VII. B. *Silver Staining*.

Appendix B

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 CometAssay™ is performed on the samples as indicated in Section VII: *Assay Protocol*. The staining step is omitted, samples are fixed with ethanol, air 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 over-night 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- or fluorescein-conjugated.

4. Wash sample 3 times with PBST for 5 minutes each.

5. If a biotinylated secondary antibody was used, incubate with streptavidin-fluorescein (Cat# 4800-30-14) diluted 1:300 in PBS (or streptavidin coupled to the 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, such as PI that does not interfere with the fluorophore.

The product accompanying this document is intended for research use only and is not intended for diagnostic purposes or for use in humans.

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