

CometAssay® Silver

**Reagent Kit for
Single Cell Gel Electrophoresis Assay
and
Silver Staining**

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and Silver Staining**

Catalog # 4251-050-K

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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.

In this assay, 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. 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.

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.

We also 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.

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

<u>CometAssay Reagents</u>	<u>Catalog #</u>	<u>Amount</u>	<u>Storage</u>
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 dH₂O 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. 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. SYBR[®] Green 1 Staining Solution* (cat# 4250-050-05)
12. Dimethylsulfoxide
13. Tris Borate EDTA buffer

*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, Ca²⁺ and Mg²⁺ free

Dilute 10X PBS (cat# 4870-500-6) 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; the LMAgarose formulation is proprietary.

For Alkaline Comet Assay:

4. 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
dH ₂ O	49.75 ml

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

5. Alkaline Electrophoresis Solution pH >13 (200 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	8 g
500 mM EDTA, pH 8	2 ml
dH ₂ O (after NaOH is dissolved) q.s. to:	1 liter

Use of freshly made solution is recommended. Prechill at 4°C.

For Neutral Comet Assay:

6. 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.

7. DNA Precipitation Solution

Prepare a 10 ml stock solution of 7.5M Ammonium Acetate:

NH ₄ Ac (mol. wt. = 77.08)	5.78 g
dH ₂ O (after NH ₄ Ac is dissolved) add to:	10 ml

For 50 ml of DNA precipitation solution combine:

7.5 M NH ₄ Ac (mol. wt. = 77.08)	6.7 ml
95% EtOH (reagent grade)	43.3 ml

8. Silver Staining Solutions

A. Fixation solution

Prepare immediately before fixation. Mix per sample:

10X Fixation Additive (cat# 4254-200-05)	10 µl
dH ₂ O	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 dH₂O 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 ₂ O	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 ₂ O	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.8.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 1×10^5 cells per ml combined with 500 µl of LMAgarose will provide the correct agarose concentration and cell density for optimal results when plating 50 µl per sample.

A. Suspension Cells

Cell suspensions are harvested by centrifugation. Resuspend cells at 1×10^5 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 1×10^5 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 1×10^5 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 ($1-2 \text{ mm}^3$), 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 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 KMnO_4 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.

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 1×10^7 cells/ml in 10% (v/v) dimethylsulfoxide, 40% (v/v) medium, 50% (v/v) fetal calf serum.
3. Transfer aliquots of 2×10^6 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 CometAssay®.

VII. Assay Protocol

A. Comet Assay

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.

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 1×10^5 /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 micro-centrifuge 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 ²⁺ and Mg ²⁺ free) at 1×10^5 /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. 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 Alkali 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.
11. Proceed to section C. *Silver Staining*.

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 1×10^5 /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 1×10^5 /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 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.
11. Proceed to section C. *Silver Staining*.

C. Silver Staining

1. Cover the sample area with 100 µl of Fixation solution prepared in section V.8.A, *Reagent Preparation*.
2. Incubate for 20 minutes at room temperature.

3. Rinse in dH₂O for 30 minutes. (Removal of all residual acetic acid is essential.)
4. Cover sample area with 100 µl of Staining Solution prepared in section V.8.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 dH₂O.
8. Air dry and store in the dark.

VIII. Warning/Safety

The final Silver Staining solution (prepared in section V, step 8 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.

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 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 4 °C.
	Intracellular activity.	Keep cells on ice and prepare cell samples immediately before combining with molten LMAgarose.
Majority of cells in the untreated control sample have small to medium comet tails.	LMAgarose too hot.	Cool LMAgarose to 42 °C before adding cells.
	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. Sample was not processed correctly.
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.

PROBLEM	CAUSE	ACTION
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 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. 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

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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
4250-050-ESK	CometAssay® Starter Kit	each
4250-050-ES	CometAssay® Electrophoresis System	1 system
4250-050-K	CometAssay® Kit	50 samples
4252-040-K	CometAssay® HT	40 samples
4254-200-K	CometAssay® Silver Staining Kit	200 samples
4253-096-K	CometAssay® Kit 96 Wells	96 samples

PARP Assay Kits:

Catalog #	Description	Size
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	Fpg Kit	8-oxoguanine, DNA containing formamidopyrimidine moieties	75 samples
4040-100-FM			100 samples
4055-100-FK	T4-PDG Kit	Cis-syn isomers of cyclo-butane pyrimidine dimers	75 samples
4055-100-FM			100 samples
4065-100-FK	cv-PDG Kit	Cis-syn and trans-syn isomers of cyclobutane pyrimidine dimers	75 samples
4065-100-FM			100 samples
4130-100-FK	hOGG1 Kit	8-oxoguanine, DNA containing formamidopyrimidine moieties	75 samples
4130-100-FM			100 samples
4100-100-FK	UVDE Kit	Cyclobutane pyrimidine dimers, (6-4) photoproducts	75 samples
4100-100-FM			100 samples

Catalog #	Description	Damage Recognized	Size
4045-01K-FK	Endonuclease III Kit	Thymine Glycol, 5,6-dihydro-thymine, urea, 5-hydroxy-6-hydrothymine, 5,6-dihydro-uracil, alloxan, 5-hydroxy-6-hydrouracil, uracil glycol, 5-hydroxy-5-methylhydantoin, 5-hydroxycytosine, 5-hydroxy-uracil, methyltartonylurea, thymine ring saturated or fragmentation product	75 samples 100 samples

DNA Damage Antibodies:

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

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

XIII. 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 1×10^5 /ml with molten LMAgarose (at 37°C) at a ratio of 1:10 (v/v) and immediately pipette 50 μ l 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 μ l
Cells in 1X PBS (Ca⁺⁺ and Mg⁺⁺ free) at 1×10^5 /ml 50 μ l

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 $\leq 45^\circ\text{C}$ 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.

11 Proceed to section C. *Silver Staining*.

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 com-parative 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
dH ₂ O	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
dH ₂ O (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 streptavidin-fluorescein (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.

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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