TREVIGEN® Instructions

For Research Use Only. Not For Use In Diagnostic Procedures

CometAssay® 96

Reagent Kit for Higher Throughput Single Cell Gel Electrophoresis Assay (96-well)

Catalog # 4253-096-K

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Table of Contents

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® 96 uses our exclusive 96-well 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 times and allows for 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. 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, to monitor assay conditions and verify reproducibility between separate runs. Trevigen also provides SYBR® Green I[1] for DNA visualization by epifluorescence microscopy, providing improved sensitivity compared to ethidium bromide.

We recommend the use of Trevigen's CometAssay® Electrophoresis System (cat# 4250-050-ES), designed to eliminate known causes of assay variability when using an Alkaline Electrophoresis Solution pH > 13. 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.

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 this 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.

[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.13 for complete licensing terms. Use of this reagent outside of the scope of these terms is not endorsed by Trevigen,Inc.

III. Materials Supplied

Component	Catalog #	Amount	Storage
Lysis Solution	4250-010-01	100 ml	Room temp.
Comet LMAgarose (LMA)	4250-050-02	15 ml	4°C
Trevigen CometSlide™ 96	4253-096-03	1-96 well slide	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 pipettes
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[1] (cat# 4870-500-6)
3. 95% Ethanol (reagent grade)
4. TE Buffer (10 mM Tris (pH 7.5), 1 mM EDTA)
5. NaOH Pellets
6. 0.5 M EDTA (pH8.0)

Optional reagent:

7. Dimethylsulfoxide

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, sold separately).

2. Lysis Solution

Per slide (96 samples per slide) prepare enough to cover all samples. Slide can be laid flat and sample areas covered with the Lysis Solution.

Lysis Solution (cat # 4250-010-01)	50 ml
DMSO	5 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. 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. Re-staining of slides is not recommended. Anti-fade solution is stored at -20°C for one month. Darkening of solution may occur.

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
dH_2O	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
dH_2O (after NaOH is dissolved) q.s. to:	1 liter

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

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 66–133 cells per CometSlide™ sample area. Using 30 µl of a cell suspension at 1 x 10^5 cells per ml combined with 300 µl of LMAgarose will provide the correct agarose concentration and cell density for optimal results when spreading 10 µl per well.

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

Adherent Cells
Methods of harvesting may need optimization to avoid introduction of additional DNA damage. In some cases, enzymatic digestion may be preferred to mechanical harvesting by rubber policeman, since clumps of cells may generate overlapping tails. One option is to trypsinize cells with Trypsin-EDTA (0.25% Trypsin, 1 mM EDTA). First, wash the monolayer of cells with sterile PBS, pre-warmed to 37 °C. Add enough Trypsin- EDTA to coat entire monolayer. Incubate flask at 37 °C for 2 minutes or when cells easily become detached upon tapping of flask. Then add 10 ml of complete media (containing fetal bovine serum) to inactivate trypsin. Transfer cells and medium to centrifuge tube, perform a cell count, and then the pellet cells. Wash once in ice cold 1X PBS (Ca^{++} and Mg^{++} free). Resuspend cells at 1 x 10^5 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), 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^{++} and Mg^{++} 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^{++} 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 10^5 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 a set of suspension cell preparations containing different levels of DNA damage to standardize comet assay methods between individual users, different runs, and between laboratories for alkaline electrophoresis conditions (cat# 4256-010-CC).

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.

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^{++} and Mg^{++} free) and proceed with CometAssay®.

VII. Assay Protocol

The 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.* phospho-glycols, 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, including incubations and electrophoresis step. 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.

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 10^5/ml with molten LMAgarose (at 37°C) at a ratio of 1:10 (v/v) and immediately pipette 10 µl onto the CometSlide™. If necessary, use the side of a pipette tip to spread agarose/cells over sample area to ensure complete coverage. 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 10 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)	300 µl
Cells in 1X PBS (Ca^{++} and Mg^{++} free) at 1 x 10^5/ml	30 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 minutes. A 0.5 mm clear ring appears at edge of each CometSlide™ area. Increasing the gelling time to 30 minutes improves adherence of samples in high humidity environments.
5. Immerse slide flat in prechilled Lysis Solution and leave on ice, or at 4°C, for at least 30 to 60 minutes.
6. Drain excess buffer from the 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 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 slide in electrophoresis slide tray and cover with Slide Tray Overlay. Set power supply to 21 volts and apply voltage for 40 minutes. (If not using an ES unit, see the Appendix.)
9. Gently drain excess electrophoresis solution, immerse twice in dH_2O for 5 minutes each, then once 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. Place 20 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 slide to dry completely at room temperature in the dark.

12. View slide by epifluorescence microscopy. (SYBR® Green I's maximum excitation and emission are, respectively, 494 nm and 521 nm. A fluorescein filter is adequate).

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 accumulated DNA damage, migrating fragments (comet tails) from nucleoids (comet heads) 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.

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 50 cells should be scored per sample.

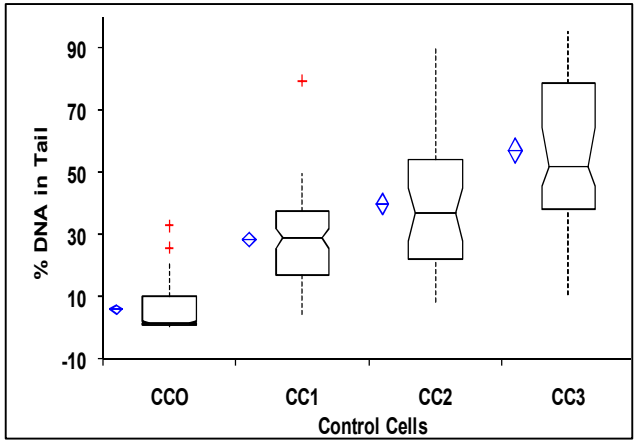
There are several image analysis systems that are suitable for quantitation of CometAssay® data. The more sophisticated systems include the microscope, camera and computer analysis package. These systems can be set up to measure the length of DNA migration, image length, nuclear size, and calculate DNA 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:

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

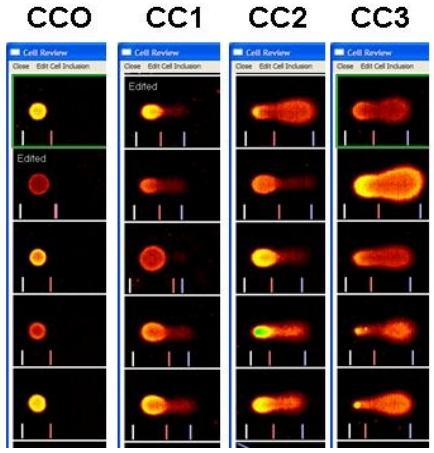


Figure 1b: Examples of comet tails for each population.

IX. References

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

Catalog #	Description	Size
4250-050-ESK	CometAssay® Starter Kit	each
4250-050-ES	CometAssay® ES	each
4250-050-K	CometAssay® Kit	50 samples
4252-040-K	CometAssay® HT	40 samples
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

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 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. 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.

PROBLEM	CAUSE	ACTION
Cells in LMAgarose did not remain attached to the CometSlide™ (cont.)	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.
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.
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 up to 1 hour for alkaline electrophoresis. Increase time of electrophoresis when running at cold temperatures.

XII. Appendix

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
dH_2O	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_2O (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 6.

SYBR® Green I nucleic acid gel stain licensing terms:
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Trevigen, Inc.
8405 Helgerman Ct. Gaithersburg, MD 20877
Tel: 1-800-873-8443 • 301-216-2800
Fax: 301-560-4973
e-mail: info@trevigen.com
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